Lincoln University
Te Whare Wānaka o Aoraki
CHRISTCHURCH•NEW ZEALAND

Lincoln University Digital Dissertation

Copyright Statement

Caucasian clover (*Trifolium ambiguum*) rhizobia persists in New Zealand soils for over 42 years

A Dissertation

submitted in partial fulfilment

of the requirements for the Degree of

Bachelor of Agricultural Science

with Honours

at

Lincoln University

by

Thomas William Steven Ley

Lincoln University

2017

Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Agricultural Science (hons)

.

Caucasian clover (*Trifolium ambiguum*) rhizobia persists in New Zealand soils for over 42 years.

by

Thomas William Steven Ley

Most legume species can fix atmospheric N_2 via bacteria (rhizobia) in root nodules, thus allowing them to colonise low nitrogen soils. The South Island of New Zealand's high country is made up of highly weathered low fertility soil that experiences frequent snow events in winter thus providing an unsuitable climate for most pasture species. Caucasian clover a winter dormant perennial clover from the Caucasus region of Eurasia has been shown to have potential in New Zealand soils, especially the high country. However, Caucasian clover has a specific rhizobia requirement, it only forms functional nodules with rhizobia from its native sites. These rhizobia do not occur naturally in New Zealand and must be supplied by inoculum. The first objective of this study was to determine if the Caucasian clover rhizobia applied as inoculum over a period of 2-42 years has persisted in New Zealand soils

Caucasian clover plants grown at the field sites were nodulated. When grown in a controlled environment, Caucasian clover plants were nodulated in soil from each of the field sites. The ERIC PCR banding patterns indicated similar genotypes over all field sites except for two isolates, as expected from Caucasian clover inoculant. Using phylogenetic analysis trees of 16S rRNA the genus of the isolates was confirmed as *rhizobium*. The *nod*A phylogenetic tree confirmed the species as *Rhizobium leguminosarum* and the *nif*H tree confirmed the biovar as *Rhizobium leguminosarum biovar trifolii*.

The second objective of this study was to determine if the Caucasian clover plants in high country soils are deficient in nutrients other than nitrogen thus limiting nodule and biomass production. Full nutrient (no nitrogen) fertiliser was applied to Caucasian clover plants at Craigieburn site in autumn

and visually assessed in spring, resulting in an increased biomass and nodule production in fertilised plants.

In conclusion, the Caucasian clover planted with specific rhizobia at different sites, aged 2-42 years, were shown by the phylogenetic analysis and ERIC-PCR to have persisted in the soil. Caucasian clover plants fertilised at the Craigieburn site were limited in non-nitrogen nutrients and showed an increased biomass and nodule production after fertiliser was applied.

Keywords: Symbiosis, nodulation, nitrogen fixation, high country, genotypic characterisation, *16S rRNA, nod*A, *nif*H, phylogenetic analysis, rhizobia specificity, *Rhizobium leguminosarum bv. trifolii*.

Acknowledgements

First and foremost, I would like to thank my supervisor Mitchell Andrews for first cultivating my interest in rhizobia in my first year of University and for taking me on as an honour student. Without his constant encouragement, funding, guidance, patience and understanding I would have never completed this project.

Secondly, I would like to thank Nguyen Tuan Dung 'Zung' for his work in the laboratory with gene work and teaching me its processes.

To the academic staff I would like to thank Alistair Black for driving during the initial trips to Craigieburn and Mesopotamia station, supplying the Caucasian clover seed and providing good advice at each harvest. Special thanks go to Dick Lucas for identifying Caucasian clover at each site and providing help with site history for the materials and methods section.

To Audrey Desaintmichel, thanks for providing data on acetylene reduction and DNA isolates from the Iverson field trials. With this help, the conclusion and discussion for this project became much stronger.

Additionally, I would like to thank Brent Richards and the Lincoln nursery team for supplying 'no nitrogen' potting mix and 'no nitrogen' fertiliser for the second trip.

To my friends thank you for helping with other school work throughout the year so I had time to work on my honours project. In particular, I would like to thank Alvand Azimi, Samuel Whitely and Mauritz de Jager for helping me stay sane and reviewing my writing.

To my family I would like to thank everyone of you for your support during the rough times and your belief that I could complete this project.

I dedicate this to my late grandfather James 'Jim' Steven for sparking my interest in agriculture and Lincoln University to begin with.

Table of Contents

List of Tables

List of Figures

Chapter 1

General Introduction

Legumes play a key role in pastoral agriculture by supplying high metabolizable energy/protein feed for grazing animals and supplementing the soil with nitrogen fixed from the atmosphere (Lüscher et al., 2014). Nitrogen fixation occurs in root nodules of a legume once it forms a symbiotic relationship with specific soil bacterial species, collectively known as rhizobia. These nitrogen-fixing root nodules allow legumes such as clovers (*Trifolium sp*.) beans (Common bean/*Phaseolus vulgaris* and field bean/*Vicia faba*) peas (*Pisum sativum*) or lucerne (*Medicago sativa*) to thrive in soil nitrogen deficient areas if other environmental factors (suitable climate, moisture and nutrient availability) are available for growth (Raven, 2010; Andrews, Raven & Lea, 2007). Symbiosis and nitrogen fixation is very energetically taxing costing 16 adenosine triphosphate (ATP) moles to fix one molecule of nitrogen (Hubbell & Kidder, 2009). Legumes vary greatly in their specificity for rhizobial symbionts. The nodulation process for almost all legumes studied is usually initiated by the legume, by producing a mix of compounds, mainly flavonoids, which induce the synthesis of *nod*D protein in rhizobia (Wang et al., 2012; Downie, 2014). Different legumes produce diverse types/mixes of chemical signalling compounds and thus attract different rhizobia.

White clover is a preferable legume for most New Zealand pastures due to its ease of growth and high nutritive value, however, it has failed to persist in New Zealand's dry hill country (Brock, Caradus and Hay 1989). Multiple alternative legume species (lucerne, *Lupinus polyphyllus* etc) have since been introduced for stock feed, but varied successes due to issues with establishment and persistence (Black et al., 2014; Moir & Moot 2010; Scott, 2014; Wills, Trainor & Scott n.d). Difficulty particularly occurred in acidic soils for lucerne (initiates horizontal root growth) and alkaloid production in lupins deterring livestock (Moir et al., N.d; Gladstones, 1970). New Zealand's high country is classified as any land ranging between 200->2000m above sea level (New Zealand Ecological Society and the New Zealand Society of Soil Science, 1994). The high country is made up of highly weathered low fertility soil that experiences frequent snow events in winter thus providing an unsuitable climate for most pasture species.

Caucasian clover (*Trifolium ambiguum*) is a perennial forage legume that has been shown by multiple authors (Allan & Keoghan, 1994; Scott, 1998; Woodman et al., 1992) to persist and remain competitive in the high country of New Zealand, despite its slow establishment rate (Widdup, Knight & Waters, 1998). The reason for this is its competitive nature, below ground growing points and

rhizomatous growth habit, which allows it to persist in harsh environments once it has been established (Bryant, 1974; Speer and Allinson, 1985). This can be attributed to its native origin of Eastern Europe and Caucasus region making it well suited for the cold dry conditions of high mountainous areas and steppes (Taylor & Smith, 1998). Once established, Caucasian clover has a good summer yield and it fixes nitrogen to a similar rate of white clover (Black et al., 2000; Widdup et al., 2001). Thus, making it a viable replacement for white clover even in summer dry lowland conditions.

Caucasian clover has very specific rhizobia requirements, with the capability to form effective nodules only with specific strains of *Rhizobium leguminosarum bv. trifolii* from Turkey and the Caucasus region (Miller et al., 2007). In New Zealand these rhizobia do not naturally exist in the soil, this is resolved by using an inoculant on the seed prior to sowing to ensure the correct rhizobia is present. Miller et al. (2007) found that one of the common inoculant strains of Caucasian clover 'ICC105', allowed nitrogen fixation in Caucasian clover but was unable to fix nitrogen in white clover. Miller et al. (2007) attributed this to the inability of expression for a subset of two nitrogen fixation genes: *nif*(*nif*H) and *fix*(*fix*A) and possibly the *nif*B operon. This is due to an insertion of 111bp in the *nif*H/*fix*A intergenic region only being found in Caucasian clover rhizobia. However, it was concluded that the genetic bases of host-specific nitrogen fixation phenotypes are still poorly understood and requires further research (Miller et al., 2007).

Caucasian clover is stated by Allan & Keoghan (1994), Scott (1998) and Strachan, Nordmeyer &White (1994) to be persistent in New Zealand high country soil once it has been established due to its symbiotic relationship with rhizobia. The persistence of Caucasian clover in New Zealand has been measured in Black et al. (2014) on a 10-year-old Caucasian clover stand, however, the persistence of the rhizobia that form a symbiotic relationship has not been measured. The rhizobia have been shown to be highly specific for Caucasian clover (Miller, et al., 2007; Elliot et al., 1998; Berenji 2015; Beauregard, Zheng and Seguin, 2003) since it does not naturally exist in New Zealand Caucasian clover requires inoculation prior to sowing for successful establishment and growth. This leads to the assumption that if a Caucasian clover plant can persist in a low nitrogen environment such as New Zealand high country soils it is likely that the rhizobia population has persisted.

1.1 Objectives of study

The major objectives of this study were to determine 1) If the Caucasian clover rhizobia applied as inoculum has persisted in New Zealand soils over a period of 2-42 years and 2) If the Caucasian clover plants in high country soils are deficient in nutrients other than nitrogen thus limiting nodule and biomass production. These objectives were achieved by:

- Assessing Caucasian clover plants at the field sites to see if they were nodulated.
- Growing Caucasian clover plants in a controlled environment (Biotron) from field site soil of nodulated plants and surface sterilised seed.
- Genotypically characterising the isolates from grown nodules based upon their housekeeping (*16s rRNA),* nodulation (*nod*A) and nitrogen fixation (*nif*H) genes.
- Applying full nutrient 'no nitrogen' fertiliser to established Caucasian clover plants at Craigieburn field site in autumn and visually assessing the plants for nodulation and biomass production in the spring.

Chapter 2

Literature review

2.1 Legumes

The legume (Fabaceae/Leguminosae) family consists of three subfamilies of *Caesalpiniodeae*, *Mimosoideae* and *Papilionoideae*. These families contain 728 genera and 19,000 species, making the legume family the 3rd most abundant family after Asteraceae (24,000 species and *Orchidaceae* (20,000 species) (Lewis et al., 2005). Legumes commonly form symbiotic relationships with soil bacteria collectively called rhizobia to fix atmospheric nitrogen with the enzyme complex nitrogenase. However, this is host plant specific with some species not having the capacity to form functional nodules for nitrogen fixation. Examples of this can be seen in the *Caesalpiniodeae* as non-nodulation remains common, while non-nodulation is uncommonly found in the *Mimosoideae* and *Papilionoideae* subfamilies (Sprent, 2009).

The subfamily *Caesalpiniodeae* is comprised of 4 tribes; *Caesalpinieae*, *Cassieae*, *Cercideae* and *Detarieae*, encompassing approximating 2250 species with 170 genera (Sprent, 2009). Morphologically *Caesalpinioideae* are recognized by commonly having actinomorphic flowers (Sprent, Ardley and James 2017). The subfamily *Mimosoideae* has two tribes (*Ingeae* and *Mimosease*) consisting of approximately 3270 species within 80 genera (Sprent, 2009). *Mimosoideae* is typically recognized by complex flower heads or flowers arranged in a spike with prominent stamens (Sprent, Ardley and James, 2017).

The subfamily *Papiliondeae* encompasses 28 tribes containing approximately 13,800 species within 480 genera, making it the largest and most complex legume subfamily (Sprent, 2009). *Papiliondeae* are morphologically recognized by zygomorphic, pea-like flowers (Sprent, Ardley & James 2017). *Papiliondeae* is the most important legume family in regard to agriculture as it contains all clover (*Trifolium*), lucerne (*Medicago sativa*), bean (*Phaseolus vulgaris, Vicia faba*) pea (*Pisum sativum*) and lupin (*Lupinus*) families (LPWG, 2017). Species of the *trifolium* tribe within the subfamily *Papiliondea* such as *Trifolium ambiguum* studied in this thesis originated from the Caucasus region and Eastern Europe.

As of 2017, the taxonomy of legumes has changed based upon the plastid *mat*K gene sequence. There are now a proposed 6 subfamilies compared to the prior three. The six new subfamilies suggested are a *Caesalpinioideae*, *Cercidoideae*, *Detarioideae*, *Dialioideae*, *Duparquetioideae* and *Papilionoideae* (Andrews & Andrews, 2017). As of a proposed change in taxonomy by the Legume Phylogeny Working Group (LPWG), the traditional subfamily *Mimosoideae* has now been changed

from a subfamily to a distinct clade and/or tribe within the *Caesalpinioideae* (LPWG, 2017). A defining factor of the new *Cercidoideae*, *Detarioideae*, *Dialioideae* and *Duparquetioideae* subfamilies is the fact that they cannot be nodulated by rhizobia and thus no nitrogen fixation occurs.

2.1.1 Nitrogen Fixation in Legumes

One of the primary characteristics of most legumes compared to other plant species is the ability to fix atmospheric nitrogen (N_2) by a symbiotic relationship with bacteria collectively known as rhizobia (4 groups, discussed in section 2.1). Legumes evolved around 60 million years ago, and gained the ability of effective nodulation and N_2 fixation 58 million years ago (Sprent, 2007). Nitrogen fixation is of utmost importance for the agricultural sector as there is an estimated 122t N_2 gas fixed per year (Hirel et al., 2011). The benefit of nitrogen fixation is that a legume can survive in a low soil nitrogen environment assuming that other factors are favourable (Raven, 2012). The rhizobia-legume symbiotic relationship allows for nitrogen fixation by forming functional root nodules capable of hosting the rhizobia in the legume, which use the nitrogenase enzyme to convert atmospheric nitrogen (N_2) into plant-usable ammonia (NH_3).

Nodulation is driven by the rhizobia specific flavonoids released from the root of a legume and the response of the released nod factors from the rhizobia resulting in infection of the legumes' root hairs. The legumes supply a home (nodule) and energy for nitrogen fixation and maintenance for the rhizobia, while the rhizobia supplies the legume with a consistent supply of nitrogen, if conditions are suitable (with some exceptions e.g. weed legumes) (Laranjo, Alexandre & Oliveira, 2014). Nodulation does not occur if specific rhizobia are not present. Once functionally nodulated the nitrogen fixation process supplies the bulk of the nitrogen for the legume family and is adjusted accordingly with the level of nitrogen in the soil, with some exceptions (e.g. European broom). (Laranjo, Alexandre & Oliveira, 2014). Ultimately the most important genes for an effective legume-rhizobia symbiosis are the genes for symbiosis, nodulation and fixation (Laranjo, Alexandre & Oliveira, 2014).

The process of effective nodulation described by Garrett & Grisham (2013) occurs as stated: legume releases specific flavonoids, this activates genes (nod) in rhizobia allowing for contact between the rhizobia and soon to be host plants root hairs by signal factors called nod factors. The roots hairs proceed to envelop the rhizobia, trapping them; allowing the infection threads to enter the roots. The infection of the root hairs can occur through a few different methods such as entering between cracks in the root hairs, root hair infection or epidermal entry (Sprent, 2009). The infection threads allow the bacteria into root cells, causing cortical cell division. The bacteria in the cells are then packaged into the plant cell membrane, transforming the membrane into a peribacteroid membrane. Rhizobia bacteroids are then released from the infection thread to divide and form symbiosomes allowing for the creation of the nitrogenase enzyme displaying effective nodulation (equation 1).

$N_2 + 8H^+ + 8e^- + 16ATP$--- Nitrogenase enzyme(catalyst)--> $2NH_3 + H_2 + 16ADP + 16Pi$

Equation 1 Atmospheric fixation in legumes with the nitrogenase enzyme complex

Not all rhizobia impregnate nodules and fix nitrogen due to the lack of the *Nif HDK* (nitrogenase) gene. The effectiveness of a legume's nitrogen fixation is measured by the 15N abundance present in the shoots of the plant or by acetylene reduction of the nodules and roots (Andrews et al., 2011). If the concentration of 15N is closer to atmospheric value than the soil value, then the nodules are working effectively (Andrews et al., 2011; Bai et al., 2012).

The fixation process is energetically expensive and thus does not occur under stressed conditions for most legumes. When Rhizobia fixes nitrogen they require 16 moles of ATP to convert each mole of nitrogen gas into ammonia (Hubbell & Kidder, 2009) The energy for nitrogen fixation is supplied by the carbon fixed by photosynthesis of the host plant. Mineral nitrogen in the soil decreases the rate of nitrogen fixation. When there is sufficient nitrogen in the soil for plant growth nitrogen fixation shuts down (Andrews et al., 2011), likely due to its energetically expensive cost.

2.2 Rhizobia

2.2.1 Taxonomy and characterisation of Rhizobia

Rhizobia is a general collective term for bacteria present in the soil that can form atmospheric nitrogen fixing nodules on the roots of members of the Fabaceae/Leguminosae family (Graham, 2008). The most recent New Zealand rhizobia taxonomy (Weir, 2006) shows that there are currently 13 genera capable of effectively nodulating specific legumes, 11 of them are within the Alphaproteobacteria (*Rhizobium*, *Mesorhizobium*, *Ensifer*/*Sinorhizobium*, *Bradyrhizobium*, *Phyllobacterium*, *Microvirga*, *Azorhizobium*, *Ochrobactrum*, *Methylobacterium*, *Devosia*, *Shinella*) and with the remaining two within Betaproteobacteria (*Cupriavidus* and *Burkholderia*) (Weir, 2016). Both alpha and betaproteobacteria form functional nodules on species specific legumes (Graham, 2008).

Characterisation of Rhizobia is based off the 16S ribosomal RNA (*rRNA*) gene which is found in nearly all bacteria, and helps with differentiation at the genus level. The other critical factors for identity differentiation are the housekeeping genes (*atpD, glnII*, and *recA*) as well as the two genes classes for nodulation (*nod*, *nol* and *noe*) and fixation *(nif* and *fix*) (Gaunt et al., 2001). The *nod* or nodulation genes (e.g. *nod*D, *nod*ABC) control the nodulation process in legume species by the creation and excretion of Nod factors created by encoded enzymes (Downie, 1998). These genes are the *nif* or nitrogen fixation genes (e.g. *nif*HDK), and control the production of the enzyme complex nitrogenase which acts as the catalyst for the N_2 fixation process (Kaminski et al., 1998). The location of the nod

and *nif* genes on chromosomal symbiosis islands can result in lateral gene transfer causing characterisation complications and a decrease in legume productivity, however, it can also allow for rhizobia to nodulate a different host species (Finan, 2002). This is only a minor occurrence though. Rhizobia are genotypically characterised by methods such as box polymerase chain reaction (BOX-PCR) DNA fingerprinting, amplified fragment length polymorphism (AFLP), genome sequencing, DNA hybridisation, random amplified polymorphic DNA (RAPD), repetitive sequence based PCR (rep- PCR), amplified rRNA restriction analysis, and DNA sequencing based on specific genomic loci. (Vandamme et al., 1996) and 16S rRNA gene-sequence analysis (Laguerre et al., 1994; Ludwig et al., 1998).

2.2.2 Specificity in Legume-Rhizobia Symbiosis

The symbiosis between legumes and rhizobia vary in their degree of specificity. Some rhizobia seem to be very host specific. For example, *Rhizobium leguminosarum biovar trifolii* only forms nodules on *Trifolium* species (Dénarié, Debellé & Rosenberg, 1992). Other rhizobia have a broad host range. An example of this would-be Rhizobium sp. strain NGR234, which can form functional nodules with 122 genera of legume species and the non-legume *Parasponia* (Pueppke & Broughton, 1999). *Bradyrhizobium* sp. would be another example of promiscuous rhizobia as it can nodulate the majority of introduced "weed" legumes within New Zealand such as wattles (Acacia spp.), gorse (*Ulex europaeus*), European broom (*Cytisus scoparius*), tagasaste (*Chamaecytisus palmensis*) and tree lupin (*Lupinus arboreus*) (Liu, 2014).

Some legumes are very rhizobia specific, for example Caucasian clover can only have effective nitrogen fixation with *rhizobium leguminosarum bv. trifolii* from the Caucasus region (e.g. ICC105, ICC148) (Miller et al., 2007), while other hosts can have a wide range of rhizobia such as *Dipogon lignosus,* which is effectively nodulated by species of *Bradyrhizobium*, *Burkholderia* and rhizobium sp.; forming both determinate and indeterminate nodules (Liu, 2014). The climate of origin also affects the host legumes promiscuity. Legumes of tropical origins have a greater level of promiscuity than temperate origin legumes (Gu et al., 2007).

Most interactions fail between non-compatible legume species and rhizobial symbionts, because of the lack of recognition of host legume flavonoids by rhizobial *nod*D proteins. This leads to the lack of expression of the rhizobial *nod* genes, preventing the production of nod factors (nodule development trigger). In some cases, nodulation does occur, but the rhizobia do not enter the nodules or intracellular release from infection threads doesn't occur. Finally, if the legume effectively nodulates, rhizobial differentiation into bacteroids on the host plant's roots can still fail to fix nitrogen due to the lack of the correct *nif*/*fix* genes. (Miller et al., 2007).

2.2.3 Environmental effects on Rhizobia and N_2 fixation

Various environmental factors can be detrimental to the survival and productivity of legumes and their rhizobia symbionts. The main detrimental factors include water/nutrient stress, soil temperature and soil acidity/alkalinity. Soil temperature may influence root hair infection, bacteroid differentiation, nodule structure, and the functionality of the host legume's root nodule (Roughley & Dart, 1970; Roughley, 1970). High soil temperatures may result in reduced rhizobial survival in the soil and disruption of the molecular signal exchanges between legume host plants and rhizobia (Hungria & Vargas, 2000). The maximum temperature for clover rhizobia strains survival in the soil was shown by Bowen and Kennedy (1959) to range from 31.0°C to 38.4 °C, depending on species. Nodulation can also be suspended in the subsurface by high soil temperatures (Graham, 1992). If nodules are already present, high soil temperatures can decrease nodule effectiveness by decreasing nitrogenase activity (Hungria and Franco, 1993; Junior et al., 2005).

In high country soils, nutrient stress is a limiting factor due to low fertility of parent material of soils and available nutrients washing downhill or being inaccessible due to pH. The required nutrients for rhizobia symbioses is the same as for any plant, however, there is an additional need for molybdenum (Mo) as it is associated with the nitrogenase enzyme complex (molybdenum nitrogenase) which allows for the fixation of atmospheric nitrogen (N_2) (O'Hara, 2001).

Soil acidity increases the concentration of the heavy metals such as aluminium and manganese while decreasing the availability of calcium. This can decrease the growth of the soils rhizobia population (Hungria & Vargas, 2000; Dilworth et al., 2001). Aluminium toxicity stops root cell division and elongation (Ryan, Ditomaso and Kochian, 1993) while decreasing macronutrient accessibility and storage by binding pores in the soil. Moir et al. (N.d) found that in high soil-available aluminium concentrations, legumes such as lucerne or peas sent their roots sideways to avoid damage. Soil acidity has also been shown to alter the production of nodulation factors and the movement mechanisms (chemotaxis) of rhizobia resulting in lowered legume symbiosis rates due to ineffective molecular signalling. (McKay & Djordjevic, 1993).

Soil alkalinity is favoured for rhizobia production as opposed to acidity. This can be seen in Evans et al. (1988) for sub clover, as soils treated with carbonate (lime) had a greater population of R. leguminosarum bv. trifolii in the soil and in the greater frequency of nodules/g root. However, this is legume and rhizobia specific as some grain legumes decrease nodulation and growth with the addition of bicarbonate (Tang & Thompson, 1996)

Water stress negatively affects rhizobia growth, survival and ultimately N_2 fixation (Zahran, 1999). Nodule production and N_2 fixation response to water stress depend on the stage of plant

development. Water stress occurring during nodule growth has a direct effect on nodule development, making recovery in some cases almost impossible. Worrall and Roughley (1976) showed that decreasing the soil moisture from 5.5 to 3.5% drastically decreased nodulation of T. subterranean by reducing the number of infection threads produced in the root hairs. This process lowers the rhizobia population in each nodule, leading to reduced nodule function. In extreme cases, the formation of nodules stops under prolonged or severe drought conditions (Ramos et al., 2003). Water stress also affects the activity of the nitrogenase complex as shown by Sprent (1976). When the water content of the roots nodules drops below 80% there is an irreversible loss of nitrogenase activity.

Salinity affects the osmotic potential of the host's legumes roots, preventing an uptake of water (Zahran, 1999) and thus leading to the death of the host plant in extreme instances. Salt stress prevents the initiation of nodulation due to root hair curling; leading to reductions of N_2 fixation through loss of rhizobia respiration in the nodules (Laranjo & Oliveira, 2011).

2.3 Nitrogen Assimilation

Nitrogen is the essential macronutrient required in the greatest quantity for normal plant growth and phenological development. Nitrogen is required in plants for the creation of DNA, RNA, cytokinins, auxins, proteins/enzymes and ATP (Andrews et al., 2013). Nitrogen assimilation is the process of converting inorganic nitrogen into usable organic nitrogen forms for growth and metabolic processes. Nitrogen is primarily assimilated in two different inorganic forms; nitrate (NO_3^-) and ammonium (NH_4^+). The environmental conditions determine the form of which nitrogen is in the soil. Nitrate is more prevalent in aerobic soils, while ammonia is mainly found in grasslands or anaerobic conditions. Plants have been shown to prefer soil nitrate while bacteria and fungi prefer ammonium (Lea & Morot-Gaudry, 2001). Masclaux-Daubresse et al. (2010) showed that plants that were from low pH environments such as mature forest tended to uptake ammonium or amino acids, while plants suited to aerobic soils with a higher pH prefer nitrate.

Other forms of nitrogen such as amino acids, ammonia ureides, or amides can also be assimilated by the host legume after they have undergone a transformation into ammonium or nitrate.

Before nitrate can be used, it must be reduced into ammonium (Andrews et al., 2004). The initial step of reducing nitrate to nitrite (NO_2^-) is catalysed by nitrate reductase (NR). Following this reduction, nitrite reductase (NiR) catalyses the transformation from nitrite to ammonium (Campbell, 2002). Ammonium, derived either from nitrate reduction or by the nitrogen fixation in a host legume’s roots, is then transported to plant cells where it is assimilated into amino acids via the

glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway (Lea & Morot-Gaudry, 2001). Glutamine synthase attaches ammonium to a glutamate molecule to form glutamine. Glutamine then reacts with 2-oxoglutarate to form two molecules of glutamate; a reaction which is catalysed by glutamate synthase (GOGAT, otherwise known as glutamine 2-oxoglutarate aminotransférase) (Masclaux-Daubresse et al., 2010).

Depending on the plant and environmental conditions, the primary site of nitrogen assimilation can be in the roots or the shoots. Ammonium is primarily assimilated in the roots while nitrate can be in assimilated in either the root or the shoot depending on plant genotype, the concentration of nitrate in the soil solution and environmental conditions (Andrews, et al. 2004; Lea & Morot-Gaundry, 2011)

In Legumes, when there is a greater supply of nitrogenous compounds in the soil, the rate of nitrogen fixation decreases (Liu, 2014). This is likely due to the decreased energy requirements of inorganic nitrogen assimilation compared to the energy expenditure required for biological nitrogen fixation (BNF) followed by assimilation. However, Schubert (1995) stated that there is no clear evidence that N_2 fixation by legumes is limited by energy supply and suggested that instead N_2 fixation is often limited by environmental stresses.

2.4 New Zealand crop legumes

Crop legumes in New Zealand are classified as any legume that is grown for the specific purpose of providing an economic or environmental benefit. This can include grain, silage or soil improvement. Table 2.1 shows the common crop legumes in New Zealand soils and the rhizobia best suited to nodulate them effectively (provide nitrogen fixation).

Table 2.1 Crop Legume species and the different rhizobia that nodulate them. Modified from Andrews et al. (2013).

Legume	Rhizobia
Clover (Trifolii spp.)	Rhizobium leguminosarum bv. Trifolii
Lucerne (medicago sativa)	Ensifer meliloti Rhizobium

Lotus pedunculatus	Bradyrhizobium ICMP 5798, Bradyrhizobium ICMP 5942 Bradyrhizobium
Lotus corniculatus	Mesorhizobium loti
Lupins (Lupinus spp.)	Bradyrhizobium ICMP 8377 Bradyrhizobium
Field pea (Pisum sativum)	Rhizobium leguminosarum bv. Viciae
Soybean (Glycine max)	Bradyrhizobium japonicum

Rhizobia strain specificity is important for legume establishment and persistence. White clover (*Trifolium repens*) and red clover (*Trifolium pratense*) are nodulated by *Rhizobium leguminosarum bv. Trifolii* and do not require inoculant due to years of rhizobia inoculant naturalising in the soil (Andrews et al., 2013). Caucasian clover (*Trifolium ambiguum*) is nodulated by a similar Rhizobium bv. trifolii to white clover, however, due to a slight genetic difference it has a specific need for inoculation to cater for the gene difference (Miller et al., 2007).

Caucasian clover rhizobia are not found naturally in New Zealand soil and thus require seed inoculation at sowing (Miller et al., 2007). This rhizobia species can be a potential problem for further land use as the Caucasian clover rhizobia are almost identical to white clover rhizobia, however, it forms ineffective nodules on white clover (Miller et al., 2007). This results in production loss or death of the white clover that is not supplied with nitrogen fertiliser. This can be prevented by heavily inoculating the white clover seed before sowing to out-compete the Caucasian clover rhizobia (Elliot et al., 1998). This is debated by Pryor and Lowther (2004), as they showed that white clover plants sown into soil with high populations of Caucasian clover rhizobia were not significantly affected in yield or nitrogen fixation even when up to 99% of the soils rhizobia population was Caucasian clover rhizobia.

Lucerne is most effectively nodulated by *Ensifer meliloti*. Once nodulated with *Ensifer*, lucerne achieves greater rates of nitrogen fixation compared to nodulation by different strains of rhizobium (Liu, 2014). For example, *Rhizobium leguminosarum bv. trifolii* can form ineffective nodules on

lucerne. To prevent this, the International Collection of Microorganisms from Plants (ICMP) recommends Ensifer meliloti inoculant to be used. After years of inoculum being used in the soils, crop rhizobia naturalize in farmland requiring less inoculum to successfully nodulate the roots of a legume, while also providing competition for unfavourable rhizobia.

Lateral gene transfer has a very minor role in rhizobia symbiosis. Finan (2002) showed that planting *Lotus corniculatus* seed inoculated with *Mesorhizobium loti* in a remote site of New Zealand with no native rhizobia capable of forming nodules became more genetically diverse over a seven-year period by the transfer of a 500-kb "symbiosis island" from the original inoculant strain to a mesorhizobium strain present in the soil which lacked the symbiosis genes. This could be beneficial as the rhizobia are better adapted to the soil than imported varieties. This would negatively impact the growth of the lotus plant if the nodulation or fixation genes were not present. This is not likely the case as previous uninoculated lotus plants died from nitrogen deficiency.

2.5 Caucasian clover and its rhizobia symbioses

Caucasian clover (*Trifolium ambiguum*) is a highly persistent (once established) and environmentally tolerant (pH, water stress and phosphorus deficiency) rhizomatous clover species from the Caucasus region of eastern Europe. Caucasian clover was originally introduced to New Zealand in 1970 to areas where environmental restrictions prevented the growth of white clover (White, 1995; Watson et al., 1996). Caucasian clover has been shown on multiple occasions to only produce nodules capable of fixing atmospheric nitrogen from strains of *Rhizobium leguminosarum bv. trifolii* native to its area of origin (Caucasus region and Eastern Europe). (Miller, et al., 2007; Elliot et al., 1998; Berenji, 2015; Beauregard, Zheng and Seguin, 2003). Caucasian clover rhizobia can nodulate other species of clover (e.g. white clover) but only fix nitrogen with Caucasian clover. Miller et al. (2007) confirmed this by showing that all strains (e.g. ICC105, ICC148) that could fix N_2 on caucasian clover (fix+) would also nodulate white clover (*Trifolium repens*), however, this was an ineffective nodulation (fix-). The ability for Caucasian clover to be nodulated by specific rhizobia is due to the presence of an extra gene that is not found in white clover rhizobia (H. McIntyre, unpublished data). Miller et al. (2007) suspected this additional gene was linked to an insertion of 111-bp in the nifH/fixA intergenic region of the Caucasian clover rhizobia. Vincent (1974) stated that the rhizobia specificity was due to T. ambiguum being the only member of *Trifolium* host subgroup C. The problem with this level of specificity is that Caucasian clover growing in soils with a high content of *trifolium repens* rhizobia can experience ineffective nodulation, leading to the death of the host plant. There is also concern that soils with a high content Caucasian clover would also decrease white clover growth in the area (Elliot et al., 1998). Different strains of Caucasian clover rhizobia show varying levels of symbiotic ability and plasmid content depending on their plasmid rearrangements, and this allowed for

different forms of inoculum to be required for the different ploidy of Caucasian clover (diploid, tetraploid, hexaploid). However, this thesis will focus solely on hexaploid Caucasian clover rhizobia.

The original rhizobia used for inoculating hexaploid Caucasian clover was the *Rhizobium leguminosarum bv. trifolii* strain IMCP 105 (a re-isolate of the commercial strain ICMP4037b), however in the 1990s a superior strain was commercially released from Australia called ICC148(CC283b). This strain was highly recommended by Pryor et al. (1998) for use on hexaploid Caucasian clover due to its superior nodulation and growth. After the Commercial release of this strain (ICMP 148), it replaced ICMP 105 as the standard inoculant for Caucasian clover seeds prior to sowing. Seguin et al. (2001) showed the success of the new commercial inoculant as the per plant mass was 3 times greater 13 months after seeding when inoculated with ICC148 as opposed to ICC05 (also known as UMR6015).

Chapter 3

Materials and Methods

This study is a follow-on study from Audrey Desaintmichel's work on Caucasian clover samples from around Lincoln University aged from 2-19 years old.

3.1 Site history

The site history of the two Mesopotamia (43°38'11.4"S 170°53'17.1"E and 43°39'45.7"S 170°52'40.0"E) was described in Lucas et al. (1981). The Caucasian clover for the Mesopotamia sites was sown in an upland yellow-brown earth from the Cass soil set with the topsoil pH ranging from 4.9-5.5. The sites had an annual rainfall of 1000mm at an altitude of 600m above sea level. Prior to sowing the Mesopotamia station sites on September 4th, 1975, the area was dominated by browntop (*Agrostis capillaris*), sweet vernal (*Anthoxanthum odorataun*), H. pilosella, scattered fescue (*Festuca sp*.) and snow tussock (*Chionochloa sp*.). Sheep grazed the area to prepare for planting. At sowing, 4kg of inoculated (strain ICC105) lime-pelleted seed was oversown onto the both sites. The Fertilisers triple superphosphate (10-100kg P/ha) gypsum (30kg S/ha) and sodium molybdate (175g/ha) were also applied at sowing.

The Craigieburn site (43°11'29.6"S 171°41'45.8"E) was sown by aerial broadcasting with superphosphate and inoculated hexaploid seed (strain ICC148) by fixed-wing plane in the spring of 1998. Prior to sowing the landscape (soil type melanic brown soil) was dominated by degraded tussock land and browntop. The Site was prepared by hard grazing with merino sheep in the early spring.

The three Lincoln University sites (-43°38'47.7"S 172°28'05.8"E) ages ranged from 2 (Iverson 4),6 years (Iverson 2) to 19 years old (Iverson 8). The soil type at each of the sites was a Templeton silt loam. Iverson site 4 was sown as a seed crop in 2015 with the ICC148 inoculum by seed broadcasting. Iverson 2 was sown by broadcasting in 2011 into a lucerne /white clover mix and without being inoculated at sowing due to the previous history of Caucasian clover in the paddock. Iverson 8 was broadcast sown in 1998 with the commercial inoculant ICC148. Prior to sowing each of the Iverson sites, sheep were brought in to hard graze vegetation before superphosphate was applied (surface application).

3.2 Field sampling Plants and soils.

Soil and plant matter samples were taken from three different areas: Craigieburn, Mesopotamia station, and Lincoln University Iverson fields. The Craigieburn area had one harvest site (43°11'29.6"S

171°41'45.8"E Castle hill 7580, altitude 784 m) with harvest occurring on 8/03/2017 while Mesopotamia had two sites, site 1 (43°38'11.4"S 170°53'17.1"E altitude= 606m) and site 2 (43°39'45.7"S 170°52'40.0"E, altitude 534m) with the harvest occurring on 16/03/2017. Additionally, there were three sites from around the Lincoln University Iverson field collected by Audrey Desaintmichel on 24/8/2016. Caucasian clover (*Trifolium ambiguum*) plants were identified by Dick Lucas (Lincoln University) at both the Mesopotamia and Craigieburn sites before plants and soils were extracted via digging. Plant samples were taken as square turf ($15cm^3$) before being placed in labelled sealable zip lock bags. Samples were chosen based upon the presence of nodules Each of the sites had 3 samples with 3 control samples taken per site approximately 15 meters away from any Caucasian clover populations for a total of 18 samples. Sampling from the Lincoln University Iverson feild sites was carried out by Audrey Desaintmichel and this thesis is a continuation of her work.

Figure 3.1 Caucasian clover concentrated around tussock at the Craigieburn site. For scale the quadrat circles size is $1m^2$.

Figure 3.2 Mesopotamia station, site 1. Site is dominated by brown top, tussock and broom.

Figure 3.3 Mesopotamia site 2. Site is dominated by brown top and degraded tussock land.

Figure 3.4 Caucasian clover population at Mesopotamia station. Note that there is a lower population of Caucasian clover than the Craigieburn site.

3.3 Sample processing

The soil was taken from the rhizosphere of established Caucasian clover plants and non-Caucasian clover control plants, by separating it gently from roots and aboveground biomass. The soil used was then stored in resealable bags until the pots and seeds were sterilised for use.

Twenty one plastic 1.2L pots and trays were first cleaned with dish wash then placed in the laminar flow for 40 minutes UV light exposure. Following this, the pots were then cleaned with 70% alcohol to sterilize the pots before planting. Caucasian clover seed was surface sterilised to remove any inoculum by spraying seeds with 70% ethanol and left to sit in it within a sieve for 5 minutes before being washed off with autoclaved water. The seed used was Endura hexaploid Caucasian clover seed (line# ENR10AT). The Seeds were planted on the 28th of April 2017 in labelled pots containing the field soil to have an approximate population of 12 seeds per pot to give an establishment of one plant per pot (A. Black, personal communication, April 28, 2017). Absolute control pots were made by autoclaving 'no nitrogen' potting mix obtained from the Lincoln University nursery for 1.5 hours at

121°C to kill any bacteria. Seeds were planted in labelled absolute control pots after the mixture had cooled down. The 'no nitrogen' potting mix was made up of ratio of 400L:100L bark: pumice medium with osmocote 0-0-37 (150g), Horticultural lime (500g), superphosphate (150g) Micromax (150g) hydroflow (500g) added as nutrients.

Pots were organised in a randomised block design on a bench with two 300mmol/s lamps on each end of the table in a growth room in the New Zealand Biotron with an ambient CO^2 level. Each pot was covered in an equal mixture of autoclaved vermiculite and perlite. Watering occurred as required.

Figure 3.5 Caucasian clover plants just before harvesting. Each site was lined up next to its controls in the order from left to right Craigieburn site 1, Mesopotamia site 1, Mesopotamia site 2 and the three absolute controls (autoclaved no nitrogen potting mix) lined up on the right side of the bench.

3.4 Assessment of nodulation and isolation

3.4.1 Harvest and Isolation

Plants were harvested on 6th August 2017 (14 weeks after planting) to isolate bacterial strains for the current study. Plants roots containing nodules were washed with tap water to remove adherent soil. For each plant, root nodules were randomly selected and dissected from the roots. Two nodules

were selected from the first two reps of each site while one nodule was selected from the third rep for a total of 5 nodules taken per site. Due to a contamination, the absolute control plants were nodulated, thus a randomly selected nodule was dissected from each of the absolute control plants to see if the rhizobia symbiont was the same as the rest of the treatments. This gives a total of 5 nodules taken per treatment site with 3 nodules from control plants. The nodules were transferred to an ethanol and ultra-violet (UV) treated laminar flow cabinet and surface sterilised by immersion in 96 % ethanol for 5 seconds in a sterile Petri dish plate followed by immersion in 5 % commercial bleach solution (sodium hypochlorite). To remove all chemicals the nodules were rinsed with sterile water for 45-90 seconds depending on the size of the nodule. Surface sterilised nodules were squashed in sterile water then streaked onto a yeast mannitol agar (YMA) (Vincent, 1970) plate and incubated at 20 – 25 °C within a Contherm biosyn 6000cp incubator for 5 days. This was a total of 13 agar plates. After 5 days growth, a single colony was selected from each agar plate and added to yeast mannitol broth (YMB). Several individual colonies were sub-cultured 3 - 4 times to purify the rhizobia strains. Table 3.1 shows the contents of the composition of YMA and YMB.

Table 3.1 Contents of the bacterial medium for yeast mannitol broth (YMB) and yeast mannitol agar (YMA).

Medium	Contents	Weight (g/ L)
YMB	yeast extract	1.0
	mannitol	10.0
	dipotassium phosphate (K_2HPO_4)	0.5
	magnesium sulphate ($MgSO_4$)	0.2
	sodium chloride (NaCl)	0.1
YMA	YMB	as described
	calcium carbonate ($CaCO_3$)	1.0
	agar	15.0

3.4.2 DNA Extraction

A single colony of each bacterial strain from the subculture on a YMB agar plate was selected and transferred to a 1 mL suspension of YMB in a 1.7 mL Eppendorf tube and incubated at 28°C in a shaking incubator for 2 – 7 days (dependent on bacterial strain) at a speed of 100–250 revolutions per minutes (rpm). Then, 500µL of this bacterial culture was stored at 4°C in fridge short term for future subculture. DNA was extracted from the other 500µL bacterial culture using the standard Qiagen-Gentra PUREGENE® DNA Purification Kit for Gram-negative bacteria.

.

3.4.3 DNA Quality Measurement

The quality of the extracted DNA was determined using a spectrophotometer (NanoDrop™) at wavelengths of 260 and 280 nm to determine the DNA concentration and the degree of protein or RNA contamination. An optical density 260/ 280 nm ($OD_{260/\ 280nm}$) ratio value close to 1.8 indicates that the extracted DNA is of good quality/purity. An $OD_{260/\ 280nm}$ ratio value close to 2.0 indicates RNA contamination while an OD260/ 280nm ratio value lower than 1.7 usually indicates protein (or phenol or other contaminants that absorb strongly at or near 280nm) contamination. The sample's DNA was re-extracted if the quality of DNA was lacking purity. The sample was diluted to a concentration of 50 ng/ µL with GIBCO® UltraPure™ Distilled Water (DNAse and RNAse free).

3.4.4 Primers for PCR Amplification

The PCR was used to amplify gene fragments for DNA sequencing. Three genes were studied: 16S rRNA, N-acyltransferase nodulation protein A (*nod*A) and nitrogenase iron protein (*nif*H). Primers for PCR amplification with their sequences and sources are shown in Table 3.2. All primers were manufactured by Integrated DNA Technologies, Auckland, New Zealand. The desalted custom synthesised DNA oligonucleotide primers were shipped lyophilised and reconstituted to a stock concentration of 200 µM using DNAse and RNAse-free distilled water. All primers solutions were stored at -20 °C.

Table 3.2 Information on oligonucleotide primers used. Modified from Tan (2014).

Gene	Primer	Sequence (5'-3')	Reference
16S rRNA	F27	AGA GTT TGA TCM TGG CTC AG	(Weisburg *et al*., 1991)
	FGPS485F	CAG CAG CCG CGG TAA	(Young *et al*., 2004)
	R1494	CTA CGG YTA CCT TGT TAC GAC	(Weisburg *et al*., 1991)
nifH	PolF	TGC GAY CCS AAR GCB GAC TC	(Poly *et al*., 2001)
	PolR	ATS GCC ATC ATY TCR CCG GA	
nodA	nodA1	TGC RGT GGA ARN TRN NCT GGG AAA	(Haukka *et al*., 1998)
	nodA3	TCA TAG CTC YGR ACC GTT CCG	(Zhang *et al*., 2000)

A, C, G, T = standard nucleotides; M = C or A; Y = C or T; R = A or G; S = G or C; B = T or C or G; H = A or C or T; N = A or G or C or T; K = T or G

3.4.5 PCR Conditions

PCR amplifications were performed using the FastStartTM Taq DNA Polymerase kit (Roche Applied Science, Auckland). Each PCR reaction was set up according to Table 3.3. The PCR conditions were taken from published protocols for selected primer pairs and were optimised for annealing temperature and primer concentration, if required (Table 3.4).

Table 3.3 Contents of PCR master mix (Tan, 2014).

Master mix	µL/ tube
PCR Buffer 10x with $MgCl_2$	2.5
2.5 mM dNTPs	2.0
Forward Primer	1.0
Reverse Primer	1.0
FastStart™ Taq Polymerase	0.25
50 ng genomic DNA	1.0
DNAse and RNAse free distilled water	17.25
Total	25

Table 3.4 Conditions of ERIC PCR modified from Tan (2014).

PCR	Temperature (°C)	Duration	Cycle
16S rRNA	95	3 min	1x
	95	30 sec	
	65	30 sec	35x
	72	30 sec	
	72	7 min	1x
	4	∞	1x
nifH	95	3 min	1x
	94	30 sec	
	62	30 sec	35x
	72	45 sec	
	72	7 min	1x
	4	∞	1x
nodA	95	3 min	1x
	94	40 sec	
	49	40 sec	35x
	72	45 sec	
	72	7 min	1x
	4	∞	1x

3.4.6 Gel Electrophosphoresis

Gel electrophoresis PCR products were analysed using a gel electrophoresis method. PCR products were resolved by 1 % agarose gel (1 g of agarose in 100 mL of 1x TAE buffer) followed by staining with 0.5 µg ml -1 ethidium bromide (EtBr) solution and viewing under UV light. Four isolate samples were used from Craigieburn, four isolate samples were used from Mesopotamia station and eight isolate samples from Iverson fields, Lincoln University. This gave a total of 16 isolates with two 1000kb ladders. The ERIC PCR banding patterns of the isolates were then compared.

3.4.7 DNA Sequencing

PCR products of expected size were sequenced by the Bio-Protection Research Centre Sequencing Facility, Lincoln University (Canterbury, New Zealand) using Applied Biosystems ABI PRISM. DNA sequence data were obtained via Sequence Scanner v1.0 software (Applied Biosystems) and edited and assembled using DNAMAN (Lynnon Biosoft Corporation, Version 6.0).

3.4.8 Phylogenetic Analyses

DNA sequences for strains from the different studies were aligned and Maximum Likelihood (ML) trees constructed with 1000 bootstrap replications with partial deletion and an 80 % coverage cut off using Molecular Evolutionary Genetics Analysis (MEGA6) software. The MEGA6 model test was performed to select a model of nucleotide substitution and the 'best' model (lowest Bayesian Information Criterion (BIC) score) used for each gene. The most closely related Rhizobium sp. strains available on the GenBank sequence database were used for the *16S rRNA*, *nodA* , *nif*H, trees. A MEGA6 model test was preformed to select a model of nucleotide substitution and the "best" model was used for each gene. The Hasegawa-Kishino-Yano with uniform rate model was used for *16S rRNA*. The Tamura 3-parameter (T92) +G model was used for *nod*A tree while the *nif*H tree used Tamura 3-parameter model + uniform rate. Only bootstrap probability values ≥ 50 % are shown. All gene sequences used in this study are available on the GenBank sequence database. Their accession numbers (locus) are shown in the figures.

3.5 Growth and nodulation from added no nitrogen nutrient mix.

Fertiliser was applied to Caucasian clover plants at the Craigieburn site on the 15th of May 2017. The trial comprised of six fertilised 1-meter plots marked out by a circular quadrat with six no fertiliser control plants. Table 3.5 shows the fertiliser mixture applied to each of the fertiliser sites (marked by a green pole) whereas no fertiliser treatments were marked by a black pole. 'No nitrogen' fertiliser was applied to ensure nodulation was required for increased plant growth.

On October 16th, 2017, the twelve plants were visually assessed and photographed for comparison.

Table 3.5 'No nitrogen' fertiliser mix for 100L of soil (1m^3) applied to Craigieburn site 1. Mixture made by B. Richards at Lincoln Plant Nursery.

Fertiliser form	Fertiliser quantity
Osmocote (0-0-37)	90g
Horticultural lime	100g
Superphosphate	30g
Micromax	30g

Chapter 4

Results

4.1 Nodulation study:

4.1.1 Field site nodulation

Caucasian clover plants from each of the field sites were nodulated with internally pink nodules seen in figure 4.1. The Mesopotamia sites also appeared to have a lower number of nodules present compared to the Craigieburn site.

Figure 4.1 Caucasian clover nodules found at Craigieburn site (A) and Mesopotamia station (B). Hand and fingernail used for scale.

4.1.2 Biotron nodulation study

Sterilised Caucasian clover seed was sown in the New Zealand Biotron and grown for 14 weeks before being harvested and cleaned thoroughly to have nodulation assessed.

All plants from all soils were nodulated with internally pink nodules. The nodules from Mesopotamia site soils yielded numerous small pink nodules (4.2 A &B) while Craigieburn site soil samples yielded larger pink nodules that were fewer in number(4.2C).

Figure 4.2D shows the presence of nodules indicating contamination to the absolute control (no nitrogen full nutrient medium). The roots of the absolute controls had more nodules per plant as they were not nutrient or pH limited when growing. Contamination occurred around week 6-8.

Figure 4.2 Roots and root nodules of Caucasian clover (*Trifolium ambiguum*) plants grown in soil from rhizosphere of Caucasian clover from Craigieburn (C.) and Mesopotamia station (A&B) field sites for 14 weeks.Additionally, there is an absolute control (8D) made from 'no nitrogen' potting mix, that resulted in a contamination.

4.2 Genomic fingerprinting by ERIC-PCR

The sixteen *rhizobium leguminosarum* isolates were subjected to DNA fingerprinting using ERIC-PCR (figure 4.3). One dominant banding pattern was observed as well as two differing band patterns. All four *rhizobia leguminosarum sp*. isolates from the Mesopotamia sites and the four isolates from the Craigieburn field site had the same banding pattern except for column 8 (Craigieburn 2.2) due to improper isolation technique and lack of re-culturing. All Iverson field sites had the same dominant banding pattern except for Iverson 4 (2.0 & 2.2). The contaminated control samples were cultured with the other samples and measured on the initial PCR. This determined that the samples were the same, thus no further work was completed with the contaminated isolates.

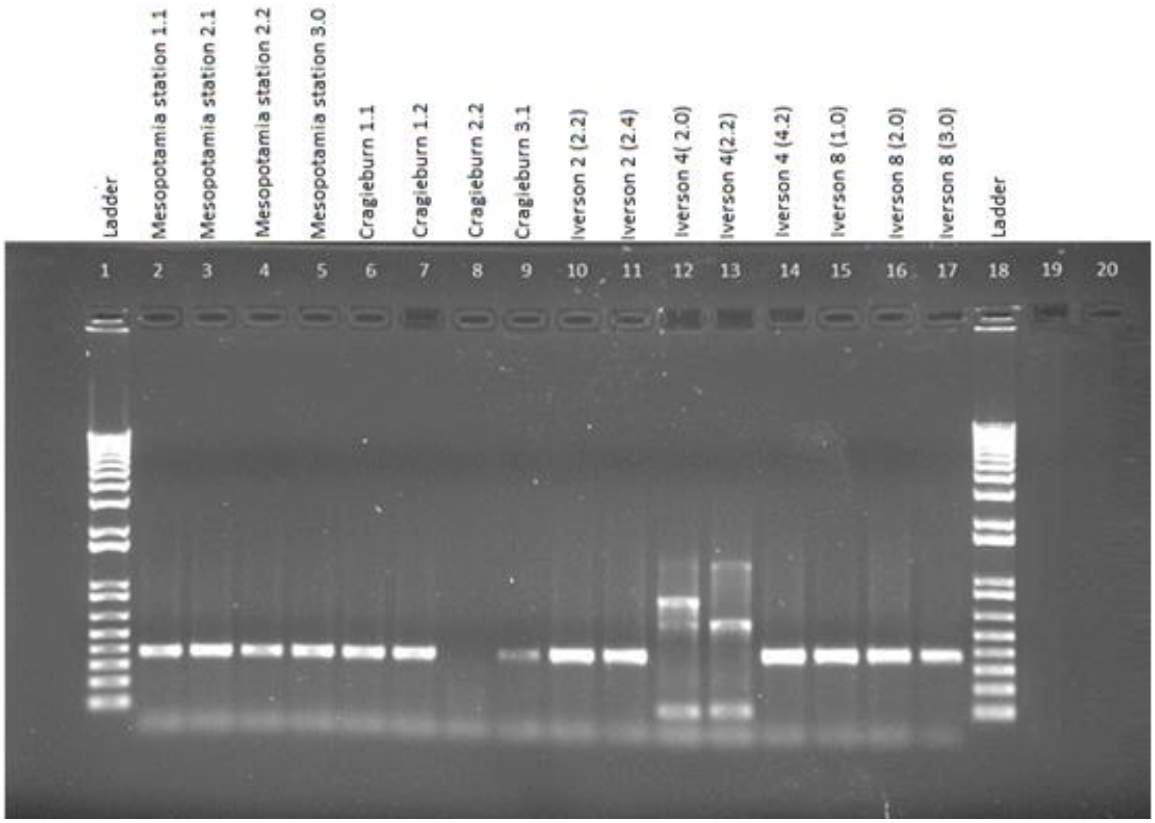

Figure 4.3 Agarose gel electrophoresis of ERIC-PCR fingerprinting patterns from genomic DNA of rhizobium leguminosarum sp. Isolates recovered from the nodules of *T. ambiguum*. Lanes 1 and 18 contain 1kb plus DNA ladder (Invitrogen, Australia); lanes 2-5 contain strains from rhizosphere Mesopotamia station isolates; lanes 6 – 9 contain Craigieburn rhizosphere isolates; Lanes 10-17 contain isolates from Lincoln university Iverson field rhizosphere soil. Isolates are indicated at the top of each lane.

4.3 Phylogenetic analysis

The phylogenetic tree for *16S rRNA* comparison of the isolate Mesopotamia station *T. ambiguum* strain TA112017 (figure 10) showed an identical similarity to *Rhizobium* spp. cb2 (LC042373, 1367 bp*), Rhizobium sp.* strain NAK 367 (MF623881, 1341 bp), *Rhizobium leguminosarum* strain pm12 (KX226358, 1382bp), *Rhizobium leguminosarum* RMCC TR0111 (KM881112, 1382bp), *Rhizobium leguminosarum bv. viciae* strain BiHB 1217 (CP022665, 5076409 bp) with no similarity to the out-group *Agrobacterium tumefaciens* CIP 43-76 (AJ389900, 1437bp). The various origins of these strains respectively are Japan (*Vicia villosa*), Kenya (*Phaseolus vulgaris*), Pakistan (*Pisum sativa*), Romania (*Trifolium repens*), in Himalayan India (*Rhizobium leguminosarum bv. Viciae*), and *Agrobacterium tumefaciens* in Lyon, France. This similarity confirmed the genus level of the Mesopotamia station strain as *Rhizobium*.

Table 4.1 Comparison of the rhizobia strains most closely related to the Mesopotamia station strain (TA112017) In a *16S rRNA* phylogenetic tree to compare similarity by using their size (base pairs), location and host legume. All strains selected were related to the Mesopotamia station *Trifolium ambiguum* strain tested (bootstrap probability ≥ 50 %).

Strain	Genbank Locus	Size (bp)	Harvest site	Legume host	Reference
Rhizobium sp. cb2	LC042373	1367	Japan	*Vicia villosa*	https://www.ncbi.nlm.nih.gov/nuccore/LC042373 - Unpublished
Rhizobium sp. strain NAK 367	MF623881	1341	Kenya	*Phaseolus vulgaris*	https://www.ncbi.nlm.nih.gov/nuccore/MF623881 - Unpublished
Rhizobium leguminosarum strain pm12	KX226358	1382	Pakistan	*Pisum sativa*	https://www.ncbi.nlm.nih.gov/nuccore/KX226358 - Unpublished
Rhizobium leguminosarum RMCC TR0111	KM881112	1382	Romania	*Trifolium repens*	(Efrose et al., 2017).
Rhizobium leguminosarum bv viciae strain BiHB 1217	CP022665	5076409	Himalayan India	-	https://www.ncbi.nlm.nih.gov/nuccore/CP022665 -Unpublished
Mesopotamia station Trifolium ambiguum (■)	TA112017	1361	Mesopotamia station, New Zealand	*Trifolium ambiguum*	This trial

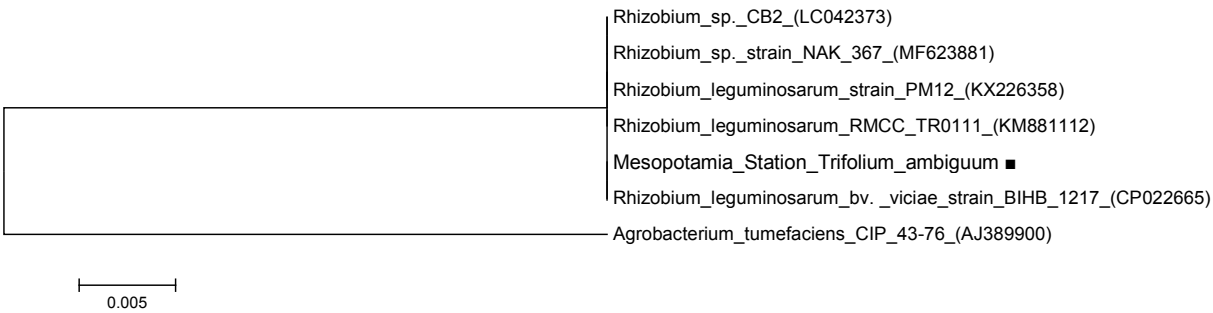

Figure 4.4 Phylogenetic tree of *16s rRNA* sequences (ca. 1361 bp) of the rhizobia isolate of *Trifolium ambiguum* from Mesopotamia station New Zealand (■) and the most closely related rhizobium type strains to it from GenBank. *Agrobacterium tumefaciens* CIP 1217 was used as an out group. This tree was constructed using the MEGA6 software with the Hasegawa-Kishino-Yano with uniform rate model. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 0.5 % sequence divergence (0.5 substitutions per 100 nucleotides).

The phylogenetic tree for *nod*A comparison of the isolate Mesopotamia station *T. ambiguum* strain TA112017 (figure 11) showed a >99% similarity with the KF680135 strain(464bp) from Russia. Only a 58% similarity to *Rhizobium leguminosarum bv. trifolii* strain GQ374362 (609bp) from Poland. Other strains used in the *nod*A tree had identical similarities among themselves but not with the Mesopotamia station strain, such as *Rhizobium leguminosarum bv. Trifolii* locus GQ374370 (609bp) and GQ374364 (609bp) originating from Lublin, Poland, while *Rhizobium leguminosarum* strain RMCC locus KM881342 (594bp) and KM881334 (594bp) were from *Trifolium repens* plants in Romania. The out-group *B. Japonicum* NZP 3209 (HE583307, 633bp) originated from *Lotus uliginosus* in Spain. *nod*A sequences confirmed the species level as *rhizobium leguminosarum*.

Table 4.2 Comparison of the rhizobia strains most closely related to the Mesopotamia station strain (TA112017) In a *nod*A phylogenetic tree to compare similarity by using their size (base pairs), location and host legume. All strains selected were related to the Mesopotamia station *Trifolium ambiguum* strain tested (bootstrap probability ≥ 50 %).

Strain	Genbank Locus	Size (bp)	Harvest Site	Legume Host	Reference
Mesopotamia station Trifolium ambiguum (■)	TA112017	540	Mesopotamia station, New Zealand	*Trifolium ambiguum*	This trial
Rhizobium leguminonsarum bv trifolii E urp CauRCR213	KF680135	464	Russia	-	https://www.ncbi.nlm.nih.gov/nuccore/KF680135 - Unpublished
Rhizobium leguminosaum bv. trifolii strain 3.6	GQ374362	609	Lublin, Poland	*Trifolium sp.*	https://www.ncbi.nlm.nih.gov/nuccore/GQ374362 - Unpublished
Rhizobium leguminosarum bv. Trifolii 9.8	GQ374370	609	Lublin, Poland	*Trifolium sp.*	https://www.ncbi.nlm.nih.gov/nuccore/GQ374370 - Unpublished
Rhizobium leguminosarum strain RM CC TR0721	KM881342	594	Romania	*Trifolium repens*	(Efrose et al., 2017)
Rhizobium leguminosarum bv. trifolii strain 3.13	GQ374364	609	Lublin, Poland	*Trifolium sp.*	https://www.ncbi.nlm.nih.gov/nuccore/GQ374364 - Unpublished
Rhizobium leguminosarum	KM881334	594	Romania	*Trifolium repens*	(Efrose et al., 2017)

strain RMCC TR0132					
B. Japonicum NZP 3209	HE583307	633	Spain	*Lotus uliginosus*	(Lorite et al., 2012)

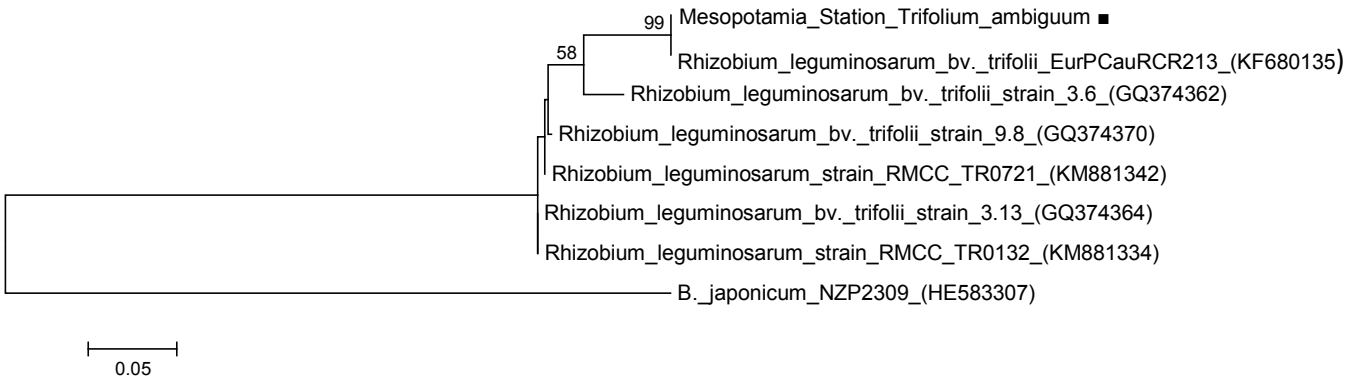

Figure 4.5 Phylogenetic tree of nodA sequences (ca. 540 bp) of the rhizobia isolate of *Trifolium ambiguum* from Mesopotamia station New Zealand (■) and the most closely related rhizobium type strains to it from GenBank. *Bradyrhizobium japonicum* NZP 2309 was used as an out group. This tree was constructed using the MEGA6 software with the Tamura 3 parameter + Gama distribution model. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides).

The phylogenetic tree for *nif*H comparison of the isolate Mesopotamia station *T. ambiguum* strain TA112017 (figure 12) show a 95% similarity with the *Rhizobium leguminosarum bv. trifolii* strain ICC105 locus EF165526 from Caucasian clover (16766bp) in Dunedin New Zealand while using the out group of B. japonicum (HM047126, 720bp, china). This Caucasian clover strain was the standard commercial inoculant used before the ICC148 superbug in the 1990s. There was a 100% similarity between Rhizobium leguminosarum bv trifolii locus KU517956 (*Trifolium hybridum*, 616bp), KU517958 (*Trifolium repens*, 649bp), KU517954 (*Trifolium repens*, 649bp), and KU517943 (*Trifolium pratense*, 617bp) all from Illinois USA. The nifH testing has confirmed the biovar level as *rhizobium leguminosarum bv. Trifolii*.

Table 4.3Comparison of the rhizobia strains most closely related to the Mesopotamia station strain (TA112017) In a *nif*H phylogenetic tree to compare similarity by using their size (base pairs), location and host legume. All strains selected were related to the Mesopotamia station *Trifolium ambiguum* strain tested (bootstrap probability ≥ 50 %).

Strain	Genbank Locus	Size (bp)	Harvest Site	Legume Host	Reference
Rhizobium leguminosarum bv trifolii strain 209	KU517956	649	Illinois, USA	*Trifolium hybridum*	(Gordon et al., 2016)
Rhizobium leguminosarum bv. trifolii strain 231	KU517958	649	Illinois, USA	*Trifolium repens*	(Gordon et al., 2016)
Rhizobium leguminosarum bv. trifolii strain 173	KU517954	649	Illinois, USA	*Trifolium repens*	(Gordon et al., 2016)
Rhizobium leguminosarum bv. trifolii strain 61	KU517943	617	Illinois, USA	*Trifolium pratense*	(Gordon et al., 2016)
Mesopotamia station caucasian clover (■)	TA112017	400	Mesopotamia station, New Zealand	*Trifolium ambiguum*	This trial
Rhizobium leguminosarum bv trifolii strain ICC105	EF165526	16766	Dunedin, New Zealand	*Trifolium ambiguum*	(Miller et al., 2007)
Bradyrhizobium japonicum strain LMG 6138	HM047126	720	Beijing, China	*Arachis hypogaea*	https://www.ncbi.nlm.nih.gov/nuccore/HM047126 - Unpublished

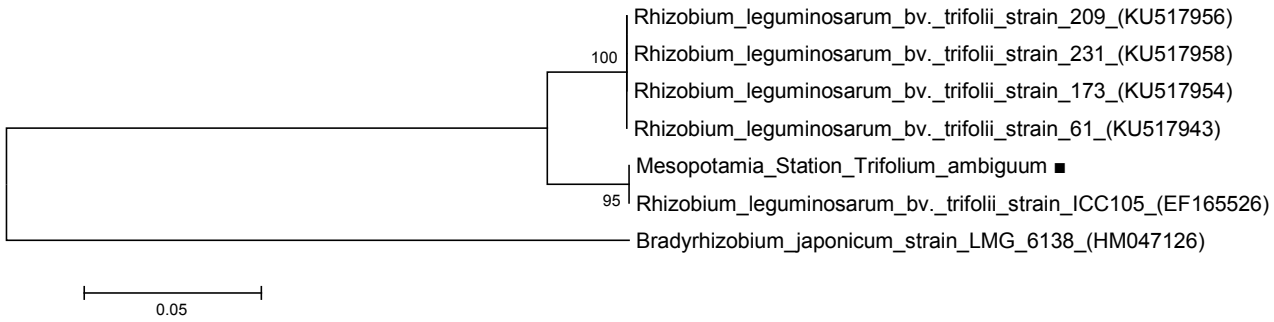

Figure 4.6 Phylogenetic tree of nifH sequences (ca. 400 bp) of the rhizobia isolate of Trifolium ambiguum from Mesopotamia station New Zealand (■) and the most closely related rhizobium type strains to it from GenBank. Bradyrhizobium japonicum LMG 6138 was used as an out group. This tree was constructed using the MEGA6 software with Tamura 3 parameter model + uniform rate. Numbers on branches are bootstrap % from 1000 replicates (shown only when ≥ 50 %). Scale bar = 5 % sequence divergence (5 substitutions per 100 nucleotides).

4.4 Growth and nodulation from fertiliser application.

Full nutrient (except nitrogen) fertiliser was applied on May 15th, 2017 to various *T. ambiguum* population at the Craigieburn site and revisited on the 16th of October for visual assessment. This allowed the plants to grow for 1.5-2 months after the winter dormancy of *T. ambiguum* has ended. Plant biomass was clearly shown to increase in the fertilised samples with the greatest biomass increased around the middle of the hill (Figure 4.7C). Nodulation also increased compared to nonfertilized plants (refer to figure 4.1 and section 4.1.1) producing numerous white/pink nodules.

Figure 4.7 Results from 'no nitrogen' fertiliser application in May on the growth of pre-established *Trifolium ambiguum* plants in Craigieburn. Black sticks represent no fertiliser treatment while green sticks show fertiliser treatment. Each fertilised quadrat is next to its non-fertilised counterpart from the same level of elevation on the hill (elevation increases with from A&B-E&F). A 1-meter quadrat circle is used for scale as well as showing the area of fertilizer application. As

Caucasian clover is dormant over the winter period this displays approximately 1-2 months of spring growth.

Figure 4.8 Increased nodulation at Craigieburn site after the addition 'no nitrogen' fertiliser (A and B).

Chapter 5

Discussion

Caucasian clover has a specific rhizobia requirement, it only forms functional nodules with rhizobia from its native sites. These rhizobia do not occur naturally in New Zealand and must be supplied by inoculum.

The major objectives of this study were to determine 1) If the Caucasian clover rhizobia applied as inoculum has persisted in New Zealand soils over a period of 2-42 years and 2) If the Caucasian clover plants in high country soils are deficient in nutrients other than nitrogen, thus limiting nodule and biomass production. These objectives were achieved by: 1) assessing Caucasian clover plants at the field sites to see if they were nodulated, 2) growing Caucasian clover plants in a controlled environment (Biotron) from field site soil of nodulated plants and surface sterilised seed, 3) genotypically characterising the isolates from grown nodules based upon their housekeeping (*16s rRNA)*, nodulation (*nod*A) and nitrogen fixation (*nif*H) genes and 4) applying full nutrient 'no nitrogen' fertiliser to established Caucasian clover plants at the Craigieburn field site in autumn and visually assessing the plants for nodulation and biomass production in the spring.

5.1 Nodulation studies

At the initial harvest, Caucasian clover plants at both Mesopotamia station and Craigieburn were nodulated with pink nodules. Nodules from Caucasian clover plants grown in soil from the Craigieburn and Mesopotamia station sites under controlled conditions were also nodulated with pink nodules. Functional nodules are red or pink internally due to the presence of leghaemoglobin. Leghaemoglobin is a red, iron-rich, oxygen and nitrogen carrier which occurs in root nodules and is highly correlated with the activity of the nitrogenase enzyme complex activity, which results in the nodules' red colour (Dakora, 1995). These nodules were assumed to be functional, however this needs to be tested by acetylene reduction to confirm the nitrogen fixation of each plant (Andrews et al., 2011).

Plants grown in Craigieburn soils under controlled conditions produced larger nodules than plants grown in Mesopotamia station soils with the same conditions. The larger nodule size may be from a superior rhizobium inoculant used at Craigieburn compared to the Mesopotamia sites. The inoculant used at the Craigieburn site is suspected to be ICC148, while ICC105 was used at the Mesopotamia station sites.

The effectiveness of a rhizobia strain can be determined by the number of plants nodulated. Pryor et al. (1998) found that when supplying Caucasian clover plants with the inoculum strain ICC148, the number of nodulated seedlings increased by 23-49% compared to the seedlings using the standard inoculum (ICC05).

Additionally, Chen & Thornton (1940), found that the average volume of active bacterial tissue is approximately three times as great in 'effective' as in 'ineffective' nodules due to lack of early growth in nodules with ineffective rhizobia strains. This results in larger nodules. Seguin et al. (2001) revealed the success of the new commercial inoculant for nitrogen fixation by showing that the per plant mass was 3 times greater 13 months after seeding when inoculated with ICC148 as opposed to ICC05

Prior to harvest, the Caucasian clover plants grown in Craigieburn soils under controlled conditions had the greatest above ground biomass of any of the field site (Figure 3.5). This result could be related to two possibilities: 1) the ICC148 inoculum produces greater growth than plants inoculated with ICC105, or 2) there is a lower number of rhizobia in the soil at sites using the original inoculant (ICC105), as degradation of the rhizobia population can occur over time (Mesopotamia sites = 42 years old, Craigieburn site = 19 years old). To confirm either of these hypotheses, the effectiveness of each strain on biomass production needs to be tested as well as the rhizobia population in the soil of the site.

Contamination of the absolute controls was seen in week 8 of growth. The most likely reason for this was the overwatering of the plants due to the poor soil drainage, leading to transfer of rhizobia by water overflow.

5.2 Genomic fingerprinting

The ERIC PCR was dominated by a single banding pattern indicating that the Craigieburn and Mesopotamia strains (lanes 2-9) are similar to the Iverson field strains supplied by Audrey (lanes 10-17) with the exception of lanes 12 and 13. The difference in lanes 12 and 13 from the major banding pattern is likely due to previous clover inoculum history at the site using other *Rhizobium leguminosarum bv. trifolii* strains that are different to the strain used for Caucasian clover, forming ineffective nodules. Thus, the strains used in lanes 12 and 13 need to be checked to determine if they can form functional nodules on Caucasian clover. The similarity of the banding pattern over all sites indicates that it is highly likely that the same rhizobia strain supplied by inoculum at sowing is being measured from each of the differently aged sites. This could be problematic because no negative control or comparison to the commercial inoculant was used. Further, there is a possibility that Caucasian clover rhizobia has spread throughout New Zealand.

Acetylene reduction was completed showing a magnitude of difference between the controls and isolates from the tested plants. Due to a processing error the acetylene reduction scores were drastically lower in plants grown in soils from Mesopotamia and Craigieburn than plants grown at Iverson field. However due to the same banding pattern being present at each of these sites and the internally red nodules grown under controlled conditions on each of the Caucasian clover plants from Mesopotamia station and Craigieburn soils, it is possible that the plants grown would achieve a similar nitrogen fixation to the Iverson field sites, but this would need to be tested.

Further studies could determine the performance of each of the isolates as they may surpass the current inoculant (ICC148).

5.3 Phylogenetic analysis

The phylogenetic tree for comparing the *16S rRNA* isolate of the Mesopotamia station *T. ambiguum* strain TA112017 (figure 10) showed that all strains were identical except the outgroup *Agrobacterium tumefaciens* CIP 43-76 (AJ389900, 1437bp). All isolates tested *were rhizobium leguminosarum* except *Rhizobium sp. cb2* (LC042373). Even though the strains were at species level (*leguminosarum*) the *16S rRNA* tree is only precise enough to confirm to genus level classification. Further testing is required to get species level accuracy. Due to the diverse locations of the isolates were from Japan, Pakistan, Romania, and Himalayan India, it is likely that the strains used are from commercial inoculants.

The phylogenetic tree for *16S rRNA* comparison of the isolate Mesopotamia station *T. ambiguum* strain TA112017 (figure 10) was identical to *Rhizobium* spp. cb2 (LC042373, 1367 bp*), Rhizobium* sp. strain NAK 367 (MF623881, 1341 bp), *Rhizobium leguminosarum* strain pm12 (KX226358, 1382bp), *Rhizobium leguminosarum* RMCC TR0111 (KM881112, 1382bp), *Rhizobium leguminosarum bv. viciae* strain BiHB 1217 (CP022665, 5076409 bp) with no similarity to the out-group *Agrobacterium tumefaciens* CIP 43-76 (AJ389900, 1437bp).

The phylogenetic tree for the *nod*A comparison of the isolate Mesopotamia station *T. ambiguum* strain TA112017 (figure 11) showed >99% similarity with the KF680135 strain from Russia. The Russian strains host species is not listed; however, it is suspected to be Caucasian clover due to the strain's name; *Rhizobium_leguminosarum_bv. _trifolii*_EurPCauRCR213. This similarity could be due to Southern Russia being a part of the Caucasus region of Eurasia where *T. ambiguum* grows. Zorin et al. (1976) stated that the origin of the previous *T. ambiguum* inoculum (ICC105) is from a Caucasian clover nodule in Russia. Within the *nod*A tree, each of the isolate strains was classified as *rhizobium leguminosarum bv. Trifolii*. Due to the *nod*A test only being specific or powerful enough to confirm species level, further testing is required to confirm biovar level. The 58% similarity between the

strains from Mesopotamia station strain (TA112017) and the strain from Lublin, Poland (GQ374362) are significantly different indicating that the Polish GQ37436 strain is unable to form nodules on *T. ambiguum* plants. The strains from Poland (GQ374362, GQ374370 and GQ374364) and Romania (KM881342 and KM881334) are not significantly different, indicating that they are capable of nodulating the host Trifoliuum species of other countries strains.

The phylogenetic tree for *nif*H comparison of the isolate Mesopotamia station *T. ambiguum* strain TA112017 (figure 12) showed 95% similarity with the *Rhizobium leguminosarum bv. trifolii* strain ICC105 (locus EF165526) from Caucasian clover (16766bp) in Dunedin, New Zealand (Miller et al 2007). The ICC105 strain was used as an inoculant for Caucasian clover at the Mesopotamia site in 1975 (Lucas et al., 1981) before the current inoculant ICC148 was introduced in 1998 (Elliot et al., 1998; Pryor et al., 1998). With this level of similarity to the commercial inoculum used at the initial sowing of the Mesopotamia sites, the rhizobia are confirmed at species level as *rhizobium leguminosarum biovar trifolii*. Thus, this confirms that the rhizobia of the Caucasian clover have persisted at the Mesopotamia sites for 42 years. There was a 100% similarity between Rhizobium *leguminosarum bv. trifolii* locus KU517956 (*Trifolium hybridum*, 616bp), KU517958 (*Trifolium repens*, 649bp), KU517954 (*Trifolium repens*, 649bp), and Ku517943 (*Trifolium pratense*, 617bp) all from Illinois, USA (Gordon et al., 2016). The Mesopotamia station *T. ambiguum* strain was not significantly different to this group. The difference between these groups may be the reason that Caucasian clover plants cannot fix nitrogen with strains of Rhizobium *leguminosarum bv. trifolii* from sites that do not originate from the Caucasus region. Miller et al. (2007) stated that this difference was due to an insertion of 111bp in the *nif*H/*fix*A intergenic region only being found in Caucasian clover rhizobia, thus causing its specificity. This needs to be verified by further testing.

5.5 Growth and nodulation from fertiliser application.

The application of full nutrient 'no nitrogen' fertiliser mix increased the biomass production and nodulation of Caucasian clover plants at the Craigieburn site, thus showing that the plants were nutrient-limited. Because a full nutrient mix was used, it is not possible to confirm the nutrient deficiency that has been corrected by the fertiliser application without soil testing at the site. High country soils are typically nutrient-limited due to the low fertility of the parent material of soils and because of available nutrients washing downhill or being inaccessible due to pH. The most likely nutrient deficiencies at the Craigieburn site prior to the application of 'no nitrogen' full nutrient mix may have been sulphur (seen in figure 3.1 as yellowing in young leaves), molybdenum or phosphorus and/or may be related to a non-optimal soil pH. Before discussing possible nutrient deficiencies, it should be noted that long term shortage of any essential nutrients will reduce nitrogen fixation by

lowering the growth of the host plant (Ball, 1969). Additionally, mineral nutrition affects both the rhizobia and the host legume.

Nitrogen was not likely a primary limiting factor because the plants at the site were already established and nodulated. However, since they did not have sufficient access to other elements, nitrogen fixation was likely decreased. Therefore, nitrogen may have been indirectly limiting plant growth.

Molybdenum is essential for nitrogen fixation as it is used in the nitrogenase complex (O'Hara, 2001). Molybdenum can be fixed to other elements in acidic soils, but can become available by the addition of lime or more molybdenum. It is unlikely that the Caucasian clover plants at Craigieburn were deficient in molybdenum due to the lime stone-based parent material.

Many of New Zealand's South Island high country soils are severely deficient in sulphur, leading to the reduced persistence of legumes in a pasture sward (Craighead et al., 1990; Moir & Moot, 2010). Because no additional sulphur inputs are added naturally to the Craigieburn soil (which is too far from ocean), it is highly likely that the Craigieburn plants are deficient in sulphur (seen in figure 3.1).

Phosphorus deficiency is likely for clover because New Zealand's high country soils are commonly low in phosphorus, due to the parent material being low in apatite minerals (McLaren & Cameron 1996). High country soils commonly contain high levels of allophane; a clay mineral that adsorps phosphorus, thus preventing its uptake by plants. This suggests that the Craigieburn site may be deficient in phosphorus, but this needs to be confirmed through soil and plant testing.

Due to the melanic brown soil composition at Craigieburn, this soil was not limiting for plant growth due to low pH or aluminium toxicity. This can be seen in figure 4.8A, by the absence of horizontal rooting behaviour. Melanic soil has a high proportion of its parent material made from limestone. Even in areas with a low pH, it is unlikely that the Caucasian clover is affected considerably given that Caradus et al. (2001) stated that Caucasian clover is very resistant to acidic soils, persisting down to a pH of 4.8.

As part of the New Zealand high country, Craigieburn experiences the most extreme climate of New Zealand's agricultural regions. The high country's growing period is reduced to 7-8 months per year due to the regions experiencing the hottest summers and coldest winters (Scott et al., 1985). When exposed to prolonged or severe drought, legumes stop forming new nodules (Ramos et al., 2003). This results in differing numbers of nodules depending on the time of year that assessment at the field sites occurs. This means that the number of nodules seen during plant visual assessment in October may have been influenced by climatic conditions, and not solely the fertiliser application.

Initially, the dry weight of each plant was going to be measured, however, due to clear differences between the fertilised plants and controls in biomass and nodule production, this was unnecessary so only visual assessment was carried out.

Chapter 6

Conclusion

This study has shown strong evidence that the Caucasian clover rhizobia at different New Zealand sites aged 2-42 years was shown by the phylogenetic analysis and ERIC-PCR to have persisted and assumed to remain functional. To confirm the functionality of the nodules, acetylene reduction tests should be conducted. Further studies on the rhizobia isolates could be completed to determine their performance range, as they may surpass the current inoculant. Lastly sites that have never had Caucasian clover sown in them should be measured to determine if the rhizobia have spread throughout New Zealand.

By applying full nutrient fertiliser (excluding nitrogen) to the Craigieburn high country field site and allowing 1-1.5 months of growth after winter, the biomass and nodule production of the Caucasian clover host plants showed a clear difference between the fertilised and non-fertilised plots, as expected. This indicated that Caucasian clover host plants at the Craigieburn site were limited in a non-nitrogen nutrient, however, soil testing and plant nutrient content testing is required to determine its specific deficiencies.

References

Allan, B. E., Keoghan, J. M. (1994). More persistent legumes and grasses for oversowing tussock country. *Proceedings of the New Zealand Grassland Association*, *56,* 143-l 47

Andrews, M. & Andrews, M. E. (2017). Specificity in Legume-Rhizobia Symbioses. *International Journal of Molecular Sciences*, *18*(4), 705. MDPI AG. Retrieved from http://dx.doi.org/10.3390/ijms18040705

Andrews, M., Jack, D. Dash, D. & Brown, S. (2015). Which rhizobia nodulate which legumes in New Zealand soils? *Journal of New Zealand Grasslands*, *77,* 281-286.

Andrews, M., James, E. K., Sprent, J. I., Boddey, R. M., Gross, E., & dos Reis Jr, F. B. (2011). Nitrogen fixation in legumes and actinorhizal plants in natural ecosystems: values obtained using 15N natural abundance. *Plant Ecology & Diversity*, *4(*2-3), 131-140.

Andrews, M., Lea, P. J., Raven J. A., Lindsey, K. L. (2004). Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. *Annals of Applied Biology*, *145*, 25-40.

Andrews, M., Raven, J. A., Lea, P. J. (2013). Do plants need nitrate? The mechanisms by which nitrogen form affects plants. *Annals of Applied Biology, 163*, 174–199.

Andrews, M., Scholefield, D., Abberton, M. T., McKenzie, B. A., Hodge, S. and Raven, J. A. (2007). Use of white clover as an alternative to nitrogen fertiliser for dairy pastures in nitrate vulnerable zones in the UK: productivity, environmental impact and economic considerations. *Annals of Applied Biology, 151*, 11–23. doi:10.1111/j.1744-7348.2007.00137.

Bai, S. H., Sun, F., Xu, Z., Blumfield, T. J., Chen, C., Wild, C. (2012). Appraisal of 15N enrichment and 15N natural abundance methods for estimating N_2 fixation by understorey Acacia leiocalyx and A. disparimma in a native forest of subtropical Australia. *Journal of Soils and Sediments, 12*(5), 653-662.

Ball, R. (1969). Legume and fertilizer nitrogen in New Zealand pastoral farming*. Proceedings of the New Zealand Grassland Association*, *31*, 117-126.

Beauregard, M. S., Seguin, P., Sheaffer, C. C., & Graham, P. H. (2003). Characterization and evaluation of North American Trifolium ambiguum-nodulating rhizobia. *Biology and Fertility of Soils*, *38*(5), 311–318.

Black, A. D., & Lucas, R. J. (2000). Caucasian clover was more productive than white clover in grass mixtures under drought conditions. *Proceedings of the New Zealand Grassland Association*, *62*,183-188.

Black, A. D., Harvey, A. J., Moir, J. L., & Moot, D. J. (2014). Caucasian clover responses to fertiliser, lime and rhizobia inoculation at Lake Heron Station, Canterbury. *Proceedings of the New Zealand Grassland Association, 76*, 105-109.

Black, A. D., Loxton, G., Ryan-Salter, T. P., & Moot, D. J. (2014). Sheep performance on perennial lupins over three years at Sawdon Station, Lake Tekapo. *Proceedings of the New Zealand Grassland Association, 76,* 35-39.

Berenji, S. (2015). Constraints and opportunities for lucerne (*Medicago sativa L*.), Caucasian clover (*Trifolium ambiguum M. Bieb*), and Russell lupin (*Lupinus polyphyllus L*.) in the high country of New Zealand (Doctoral dissertation, Lincoln University).

Bowen, G. D. & Kennedy. M. M. (1959). Effect of high soil temperatures on Rhizobium sp. *Queensland Journal of Agricultural Science*,*16*, 177-97.

Brock, J. L., Caradus J. R. and Hay, M.J.M. (1989) Fifty Years of white clover research in New Zealand. *Proceedings of the New Zealand Grassland Association*, *50*, 25- 39.

Bryant, W. G. (1974). Caucasian clover (*Trifolium ambiguum Bieb*.): a review. *Journal of the Australian Institute of Agricultural Science*, *40*, 11–19.

Campbell, W. (2002). Molecular Control of Nitrate Reductase and Other Enzymes Involved in Nitrate Assimilation. Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism (Vol. 12, pp. 35-48): Springer Netherlands.

Chen, H. K., & Thornton, H. G. (1940). The structure of “ineffective” nodules and its influence on nitrogen fixation. *Proceedings of the Royal Society Royal Society of London. Series B*, *129*, 208–229. https://doi.org/10.1098/rspb.1940.0036

Caradus, J. R., Crush, J. R., Ouyang, L., & Fraser, W. (2001). Evaluation of aluminium-tolerant white clover (*Trifolium repens*) selections on East Otago upland soils. *New Zealand Journal of Agricultural Research, 44*(2-3), 141-150.

Craighead, M. D., Burgess, W. B., Clark, S. A., & Duffy, R. G. (1990). Development of sunny-facing high country using different forms of sulphur fertiliser. *Proceedings of the New Zealand Grassland Association*, *52*, 203-206.

Efrose, R. C., Rosu, C. M., Stedel, C., Stefan, A., Sirbu, C., Gorgan, L. D., ... & Flemetakis, E. (2017). Molecular diversity and phylogeny of indigenous Rhizobium leguminosarum strains associated with *Trifolium repens* plants in Romania. *Antonie van Leeuwenhoek*, 1-19.

Dakora, F. D. (1995). A Functional Relationship Between Leghaemoglobin and Nitrogenase Based on Novel Measurements of the Two Proteins in Legume Root Nodules. *Annals of Botany, 75*(1), 49–54. http://doi.org/10.1016/S0305-7364(05)80008-3

Dénarié, J., Debellé, F., & Rosenberg, C. (1992) Signalling and host range variation in nodulation. *Annual Review of Microbiology, 46*, 497-531.

Dilworth, M., Howieson, J., Reeve, W., Tiwari, R., Glenn, A. (2001). Acid tolerance in legume root nodule bacteria and selecting for it. *Animal Production Science*, *41*(3), 435-446.

Downie, J. A. (1998). Functions of rhizobial nodulation genes, H.P. Spaink, A. Kondorosi, P. J. J. Hooykaas (Eds.), The rhizobiaceae molecular biology of model plant-associated bacteria, Kluwer Academic Publishers, Dordrecht, 387–402.

Downie, J. A. (2014). Legume nodulation. *Current Biology, 24*(5), 184-190.

Elliot, R. M., McIntyre, H. J., Challis, B. C., Pryor, H.N., Lowther, W. L., Ronson, C.W. (1998). Rhizobium issues affecting the contribution of caucasian clover to New Zealand pastoral agriculture. *Proceedings of the New Zealand Grassland Association*, *60*, 207– 211

Evans, J., Hochman, Z., O'Connor G E, Osborne G J. (1988). Soil acidity and *Rhizobium*: their effect on nodulation of subterranean clover on the slopes of southern New South Wales. *Australian Journal of Agricultural Research*, *38*(103),605–618.

Finan, T. M. (2002). Evolving Insights: Symbiosis Islands and Horizontal Gene Transfer. *Journal of Bacteriology*, *184(*11), 2855–2856. doi:10.1128/JB.184.11.2855-

Garrett, R. H., & Grisham, C. M. (2013). *Biochemistry*. Chapter 25 pages 877-924 Belmont, CA: Brooks/Cole, Cengage Learning.

Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A., Young, J. P. W. (2001). Phylogenies of *atp*D and *rec*A support the small subunit rRNA-based classification of rhizobia. *International Journal of Systematic and Evolutionary Microbiology, 51,* 2037–2048.

Gladstones, J. S. (1970). Lupins as crop plants. *Field Crop Abstracts, 23*, 123-148.

Gordon, B. R., Klinger, C. R., Weese, D. J., Lau, J. A., Burke, P. V., Dentinger, B., & Heath, K. D. (2016). Decoupled genomic elements and the evolution of partner quality in nitrogen-fixing rhizobia. *Ecology and evolution, 6*(5), 1317-1327.

Graham, P. H. (1992). Stress tolerance in Rhizobium and Bradyrhizobium, and nodulation under adverse soil conditions. *Canadian Journal of Microbiology,38*, 475–484.

Graham, P. H. (2008). Ecology of the root-nodule bacteria of legumes.Pages 23-58 in: Nitrogen-fixing legume symbioses. M. J. Dilworth, E. K. James, J. I. Sprent, and W. E. Newton, eds. Springer, Dordrecht, The Vol. 24, No. 11, 2011 / 1287 Netherland.

Gu, C. T., Wang, E. T., Sui XH, Chen, W. F., Chen, W. X., (2007). Diversity and geographical distribution of rhizobia associated with Lespedeza spp. in temperate and subtropical regions of China. *Archives of Microbiology, 188*, 355 -365.

Haukka, K., Lindstrom, K. & Young, J. P. W. (1998). Three phylogenetic groups of nodA and nifH genes in Sinorhizobium and Mesorhizobium isolates from leguminous trees growing in Africa and Latin America. *Applied and Environmental Microbiology*, *64*, 419-426.

Hirel, B., Thierry Tétu, T., Lea P. J., and Dubois, F. (2011). Improving Nitrogen Use Efficiency in Crops for Sustainable Agriculture. *Sustainability*, *3*, 1452-1485.

Hubbell, D.H. & Kidder, G. (2009). Biological nitrogen fixation. University of Florida IFAS Extension Publication SL16, 1–4.

Hungria, M. & Vargas, M. A. (2000). Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research*, *65*(2), 151-164.

Hungria, M., & Franco, A. A. (1993). Effects of high temperature on nodulation and nitrogen fixation by Phaseolus vulgaris L. *Plant and Soil*, *149*(1), 95-102.

Junior, M. D. A. L., Lima, A. S. T., Arruda, J. R. F., Smith, D. L. (2005). Effect of root temperature on nodule development of bean, lentil and pea. *Soil Biology and Biochemistry, 37*(2), 235-239.

Kaminski, P. A., Batut, J. & Boistard, P. (1998). A survey of symbiotic nitrogen fixation by rhizobia H.P. Spaink, A. Kondorosi, P.J.J. Hooykaas (Eds.), The rhizobiaceae molecular biology of model plant-associated bacteria, Kluwer Academic Publishers,Dordrecht, 432–460.

Laguerre, G., Allard, M. R., Revoy, F., & Amarger, N. (1994). Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Applied and Environmental Microbiology*, *60*(1), 56-63.

Laranjo, M., Oliveira, S. (2011) Tolerance of Mesorhizobium type strains to different environmental stresses. *Antonie Van Leeuwenhoek, 99*(3),651–62.

Laranjo, M., Alexandre, A., & Oliveira, S. (2014). Legume growth-promoting rhizobia: An overview on the Mesorhizobium genus. *Microbiological Research*, *169*(1), 2–17.

Lea, P. J. & Morot-Goudry, J. F., eds. (2001). Plant Nitrogen. Springer, Berlin and New York

Lewis, G., Schrire, B., Mackinder, B., Lock M. (2005). Legumes of the world. Kew. *London:* Royal Botanic Gardens.

Liu, W. Y. Y. (2014). Characterisation of rhizobia and studies on N_2 fixation of common weed legumes in New Zealand. PhD thesis. Lincoln University, New Zealand. Retrieved from: https://researcharchive.lincoln.ac.nz/handle/10182/6468.

Lorite, M. J., Videira e Castro, I., Muñoz, S., & Sanjuán, J. (2012). Phylogenetic relationship of Lotus uliginosus symbionts with bradyrhizobia nodulating genistoid legumes. *FEMS microbiology ecology, 79*(2), 454-564.

LPWG (2017). A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny. *Taxon, 66*, 44–77. 6.

Ludwig, W., Amann, R., Martinez-Romero, E., Schönhuber, W., Bauer, S., Neef, A., Schleifer, K. H. (1998). rRNA based identification and detection systems for rhizobia and other bacteria. *Plant and Soil*, *204*(1), 1–19.

Lüscher, A., Mueller-Harvey, I., Soussana, J. F., Rees, R. M., & Peyraud, J. L. (2014). Potential of legume-based grassland–livestock systems in Europe: a review. *Grass and Forage Science, 69*(2), 206–228. http://doi.org/10.1111/gfs.12124

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L., Suzuki, A. (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Annals of Botany*, *105*(7), 1141-1157.

McLaren, R. G. & Cameron, K. C. (1996). Soil Science: Sustainable production and environmental protection (Second ed., pp. 192 - 208). Australia.

McKay, I. A. & Djordjevic, M. A. (1993). Production and excretion of Nod metabolites by Rhizobium leguminosarum bv. trifolii are disrupted by the same environmental factors that reduce nodulation in the field. *Applied and Environmental Microbiology*, *59*(10), 3385-3392.

Miller, S. H., Elliot, R. M., Sullivan, J. T., Ronson, C.W. (2007). Host-specific regulation of symbiotic nitrogen fixation in Rhizobium leguminosarum biovar trifolii. *Microbiology*, *153*, 3184–3195.

Moir, J. L., & Moot, D. J. (2010). Soil pH, exchangeable aluminium and lucerne yield responses to lime in a South Island high country soil. *Proceedings of the New Zealand Grassland Association, 72*,191-196.

Moir, J., Moot, D. J., Black, A., Dick Lucas, D. (N.d). Soil pH and Aluminium Toxicity Challenges in High Country. Retrieved from http://www.lincoln.ac.nz/PageFiles/23598/2013-04-05-Soil-pH-and-Al-toxicity-Challenges-in-high-country.pdf

New Zealand Ecological Society and the New Zealand Society of Soil Science (1994). Review of South Island high country land management issues. *New Zealand journal of ecology, 18*(1) 69-81.

O'Hara, G. W. (2001). Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. *Australian Journal of Experimental Agriculture*, *41*, 417-433.

Poly, F., Ranjard, L., Nazaret, S., Gourbiere, F., Monrozier, L. J. (2001). Comparison of nifH gene pools in soils and soil microenvironments with contrasting properties. *Applied and Environmental Microbiology*, *67*, 2255-2262.

Pueppke, S. G. M. & Broughton, W. J. (1999). Rhizobium sp. strain NGR234 and R. Fredii USDA257 share exceptional broad, nested host- ranges. *Molecular Plant -Microbe Interactions*, *12*, 293-318.

Pryor, H. N., Lowther, W. L., McIntyre, H. J., & Ronson, C. W. (1998). An inoculant Rhizobium strain for improved establishment and growth of hexaploid Caucasian clover (Trifolium ambiguum). *New Zealand Journal of Agricultural Research*, *41*(2), 179-189.

Pryor, H. N. & Lowther, W. L. (2004). Caucasian clover rhizobia are not a threat to nitrogen fixation by white clover. *Proceedings of the New Zealand Grasslands Association, 66*, 285-289.

Ramos. M. L. G., Parsons R., Sprent J. I., James E. K. (2003). Effect of water stress on nitrogen fixation and nodule structure of common bean. *Pesquisa Agropecuária Brasileira ,*38 339– 347. 10.1590/S0100-204X2003000300002

Raven, J. A. (2010). Why are mycorrhizal fungi and symbiotic nitrogen-fixing bacteria not genetically integrated into plants? *Annals of Applied Biology,157*, 381–391.

Raven, J.A. (2012). Protein turnover and plant RNA and phosphorus requirements in relation to nitrogen fixation. *Plant Science*, *188–189*, 25–35.

Roughley, R. J. and Dart, P. J. (1970) Root temperature and root-hair infection of *Trifolium subterraneum* L. cv. *Cranmore. Plant Soil, 32*, 518–520.

Roughley R. J. (1970) The influence of root temperature, *Rhizobium* strain and host selection on the structure and nitrogen-fixing efficiency of the root nodules of *Trifolium subterraneum*. *Annals of Botany, 34,* 631–646.

Ryan, P.R., Ditomaso, J. M., Kochian, L.V. (1993) Aluminium toxicity in roots: an investigation of spatial sensitivity and the role of the root cap. *Journal of Experimental Botany, 44,* 437–46. doi: 10.1093/jxb/44.2.437.

Scott, D. (1998). Sixteen years of caucasian clover under contrasting managements. *Proceedings of the New Zealand Grassland Association, 60*, 115–118.

Scott, D. (2014). The rise to dominance over two decades of Lupinus polyphyllus among pasture mixtures in tussock grassland trials. *Proceedings of the New Zealand Grassland Association, 76*,47-52.

Scott, D., Keoghan, J. M., Cossens, G. G., Maunsell, L. A., Floate, M. J., Wills, B. J., & Douglas, G. (1985). Limitations to pasture production and choice of species. *Grassland Research and Practice Series, New Zealand Grassland Association Inc.,* (3), 9-15.

Schubert, S., Serraj, R., Plies-Balzer, E., & Mengel, K. (1995). Effect of drought stress on growth, sugar concentrations and amino acid accumulation in N_2-fixing alfalfa (Medicago sativa). *Journal of Plant Physiology, 146*(4), 541-546.

Seguin, P., Sheaffer, C. C., Ehlke, N. J., Russelle, M. P., & Graham, P. H. (2001). Nitrogen fertilization and rhizobial inoculation effects on Kura clover growth. *Agronomy Journal, 93*(6), 1262-1268.

Speer, G. S. & Allinson, D. W. (1985). Kura clover (Trifolium ambiguum): legume for forage and soil conservation. *Economic Botany, 39*, 165–176.

Sprent, J. I. (1976) Nitrogen fixation by legumes subjected to light and water stresses. *In* Symbiotic nitrogen fixation in plants. Cambridge University Press. Ed. P S Nutman. 405– 420.

Sprent, J. I. (2007). Evolving ideas of legume evolution and diversity: A taxonomic perspective on the occurrence of nodulation: Tansley review. *New Phytologist*, *174*(1), 11-25. https://doi.org/10.1111/j.1469-8137.2007.02015.x

Sprent J. I. (2009). Legume Nodulation: A Global Perspective. New Delhi, Wiley-Blackwell.

Sprent, J. I., Ardley, J., James, E. K. (2017). Biogeography of nodulated legumes and their nitrogen-fixing symbionts. *New Phytology. 215*(1), 40-56.

Strachan, D. E., Nordmeyer, A. H., White, J. G. H. (1994). Nutrient storage in roots and rhizomes of hexaploid caucasian clover. *Proceedings of the New Zealand Grassland Association, 56*, 77–99.

Tan, H. W. (2014). Characterisation of rhizobia associated with New Zealand native legumes (Fabaceae) and a study of nitrogen assimilation in Sophora microphylla. PhD. thesis, Lincoln University, New Zealand). Retrieved from: http://researcharchive.lincoln.ac.nz/handle/10182/6588.

Tang, C. & Thomson, B. D. (1996). Effects of solution pH and bicarbonate on the growth and nodulation of a range of grain legumes. *Plant Soil, 186*, 321–330.

Taylor, N. L. & Smith, R. R. (1998). Kura clover (Trifolium ambiguum M.B.) breeding, culture, and utilization. *Advanced Agronomy, 63*, 153–178.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K., & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews,60*(2), 407-438.

Vincent, J. (1970). A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook 15. Oxford, Blackwell.

Vincent, J. M. (1974). Root-nodule symbioses with Rhizobium. Biology of Nitrogen Fixation. *A. Quispel, ed.*

Wang, D., Yang, S., Tang, F., & Zhu, H. (2012). Symbiosis specificity in the legume– rhizobial mutualism. *Cellular microbiology, 14*(3), 334-342.

Watson, R.N., Neville, F.J., Bell, N.L., Harris, S.L. (1996). Caucasian clover as a pasture legume for dryland dairying in the coastal Bay of Plenty. *Proceedings of the New Zealand Grassland Association, 58*, 183–188.

Weir, B. (2006). Systematics, Specificity, and Ecology of New Zealand Rhizobia. PhD thesis. University of Auckland, New Zealand.

Weir, B.S. (2016). The current taxonomy of rhizobia. NZ Rhizobia website. https://www.rhizobia.co.nz/taxonomy/rhizobia Last updated: X Jan 2016

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology, 173,* 697-703.

White, I. G. H. (1995). A review of legume introduction in tussock grasslands with reference to species tolerant of low nutrient inputs. *Agronomy Society of New Zealand, 25*, 77- 85.

Widdup, K. H. Knight, T. L. and Waters, C.J. (1998). Genetic variation for rate of establishment in caucasian clover. *Proceedings of the New Zealand Grassland Association, 60*, 213–217.

Widdup, K.H., Purves, R.G., Black, A.D., Jarvis, P., Lucas, R.J. (2001). Nitrogen fixation by Caucasian clover and white clover in irrigated ryegrass pastures. *Proceedings of the New Zealand Grassland Association, 63*, 171-175.

Wills, B., Trainor, K. & Scott, D. (n.d) Legumes for South Island tussock grassland environments - an evaluation of plant survival and growth at some inland Otago and Canterbury trials. *Proceedings of the New Zealand Grassland Association*,131-142.

Woodman, R. F., Keoghan, J. M., Allan, B. E. (1992). Pasture species for drought-prone lower slopes in the South Island high country. *Proceedings of the New Zealand Grassland Association, 54*, 115–120.

Worrall, V. S. & Roughley, R. J. (1976). The effect of moisture stress on infection of *Trifolium subterraneum* L. by *Rhizobium trifolii* Dang. *Journal of Experimental Botany*,*27*, 1233–1241.

Young, J. M., Park, D-C., and Weir, B. S. (2004). Diversity of 16S rRNA sequences of Rhizobium spp. implications for species determinations. *FEMS Microbiology Letters*, *238*, 125-131.

Zahran, H. H. (1999). *Rhizobium*-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate. *Microbiology and Molecular Biology Reviews,63*(4), 968–989.

Zhang, X. X., Turner, S. L., Guo, X. W., Yang, H. J., Debello, F., Yang, G. P., Denarie, J., Young, J. P. W., Li, F. D. (2000). The common nodulation genes of Astragalus sinicus rhizobia are conserved despite chromosomal diversity. *Applied and Environmental Microbiology, 66,* 2988-2995.

Zorin, M., Hely, R W., Dear, B. S. (1976). Host-strain relationships in symbiosis between hexaploidy Trifolium ambiguum Bieb. (Caucasian clover) and strains of Rhizobium trifolii. *Australian CSIRO Division of Plant Industry field station record, 15*,63-71